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This report describes the project to fill out shell space on the third floor of Othmer Hall, the new home for the University of Nebraska-Lincoln Biological Process Development Facility. Construction is 95% completed and all research laboratories of the BPDF are functional. The 5% includes a Master Cell Banking The third floor is home to all process research capabilities, i.e. molecular biology, fermentation, cell culture, analytical methods, quality control, purification development, media prep, and two academic laboratories in the department of chemical engineering performing bioengineering research.

The contract for detailed design of the cGMP basement was awarded June 2004 and is expected to be completed by Fall 2005.

This report also describes research on the expression of an antibody against serotype A botulinum neurotoxin in Chinese Hamster Ovary (CHO). Research on new expression plasmids, development of a serum-free media, and optimization of a fed-batch process to produce the antibody against.

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Introduction

The funding for this project is comprised of two components. The first component, which represents 95% of the funding, is dedicated to filling out shell space on the third floor and basement of Othmer Hall, the new home for the Biological Process Development Facility. The other 5% is dedicated to research focused on the expression of full length chimeric antibodies against serotype A botulinum neurotoxins in Chinese Hamster Ovary (CHO) cells. This report will provide a summary of 2004-2005 activities, which includes construction of third floor research space and expression of a chimeric full-length antibody against serotype A. UNL just submitted a manuscript (4/15/04) on the BoNTA chimeric antibody project, which is incorporated into this report.

Body

Construction Project

Basement cGMP Construction Project

On December 2003 the University of Nebraska Board of Regents officially approved the cGMP basement project. A competitive bid process was initiated in February 2004 for detailed design of the cGMP facility and award was made in June 2004. This facility will be a 17,000 ft^2 with 6,000 ft^2 of clean room space and capable of producing Phase I/II material derived from bacteria, yeast, and transgenic milk. The design firm that was awarded the bid was bioKinetics of Philadelphia, PA and Davis Design of Lincoln, NE. Anticipated completion of detail design is fall 2005. This design document will be used for construction bids.

Third Floor Construction Project

Quality Control Microbiology Laboratory and Master Cell Banking Suite: The third floor is completed, except for the Master Cell Banking Suite (MCB). Half of the approximately 900 ft^2 that is under construction will be for the Quality Control Microbiology (QCM) Laboratory and the other half will be for the Master Cell Banking Suite. The QCM Lab will operate under cGMP and will provide support to MCB suite, cGMP pilot plants, and the development labs. This part of the entire project was officially started in July 2004 with the initiation of design. The BPDF is at the final stage of review and approval of final mechanical and architectural drawings. The validated isolator system, which will be the primary piece of equipment in the MCB facility, completed its Factory Acceptance Test during the week of May 16, 2005. The BPDF decided to use isolator technology for production of the MCB and Working Cell Banks (WCB) to insure that production of the MCB and WCB were completed in a validated and enclosed environment. This facility will meet both FDA and EU requirements for facility design. New completion date is projected as August 30, 2005.

Molecular Biology Laboratory (MBL): A 990 ft^2 laboratory dedicated molecular biology and strain construction.

Fermentation Development Laboratory (FDL): A 1,200 ft^2 laboratory dedicated to bacterial and yeast fermentation research. The FDL will have 10 autoclavable BioFlo III and Bioflo 3000 bench-top 5 L fermentors (New Bruinswick Scientific) and 8 Bioengineering NLF 19 L steam-in-place fermentors. The FDL will have a 150 ft^2 microbiology support laboratory for growing inoculums and storing frozen seed cultures. All of the fermentors will be computer controlled and off-gas will be sent to an off-gas mass spectrometer for metabolic analysis.

Cell Culture Development Laboratory (CCDL): The CCDL is a new capability for the BPDF and has two different laboratories. One half of the space (750 ft^2) is dedicated to mammalian cell-line development and optimization and is outfitted with biosafety cabinets and CO_2 incubators. The purpose of this space is to develop mammalian cell clones suitable for transfer into a bioreactor and process scale-up. The other half of the facility (750 ft^2) is dedicated to bioreactors for scaling-up the process from 250 mL spinners to a 200 L bioreactor. The CCDL will have 8 by 250 mL DasGip computer controlled spinner system, 4 by 3.7 L computer controlled Bioengineering ALF bioreactors integrated to a Nova 400 bioanalyzer for automated sample analysis, one 19 L computer controlled Bioengineering bioreactor and one 200 L computer controlled Bioengineering bioreactor. The off-gas from the 3.7, 19 and 200 L bioreactors will be sent to an off-gas mass spectrometer. The primary function of the CCDL will be strain development, process research and development of production technology for humanized antibodies against bioterrorism agents in CHO cells.

Purification Development Laboratory (PDL): The PDL is a 1,500 ft^2 laboratory that has the responsibility of developing the recovery and purification process at the bench-scale (7.8 to 100 mL column size) and scaling-up the process to the 1 to 2 L column size and the production of small non-cGMP lots. The PDL will have "three research

areas," bench-scale development area, central bench for routine assays, and the third is a small pilot plant area capable of processing up to 0. 5 to 1 gram research lots of material.

***Media Preparation Area*:** The third floor will have a central media preparation area that will have two 26" x 36" x 38" Primus Autoclaves and a Miele Model G7827 large capacity glassware washer. There are two small lab areas will include a chemical hood, chemical storage cabinets and dry goods storage and laboratory space to prepare buffer and media.

Dark Room and Radioisotope Room. Two small 150 ft^2 labs are designated as a dark room and a radioisotope room.

Three Bioengineering Research Laboratories. Three standard research laboratories are being constructed on the third floor. These lab spaces are planned for two faculty members in the Department of Chemical and the third laboratory will be used for the BPDF Analytical Methods Laboratory. One faculty member is working on antibody production and tissue engineering, while the second faculty member research interest is the production of complex proteins in transgenic animal. (Note: All animals for transgenic research will be housed in an animal facility in either the Departments of Animal Science or Veterinary and Biomedical Sciences on East Campus at the University of Nebraska-Lincoln.

Third Floor Office Area. The third floor will have office area for both staff and students. There will be an office area for the BPDF staff, which includes a conference room with both audio and visual conferencing capabilities.

Additional Features of the Third Floor

Utilities. The third floor will have all of the standard utilities and additional utilities that are specific to the third floor. These include chilled water (10°C), clean steam distributed in 316L stainless steel piping, a biowaste kill system that will serve both the third floor and the basement, central gas storage room for distribution of oxygen, nitrogen, carbon dioxide and helium.

Electrical. The third floor will have 3 different types of power, uninterruptible power supply (UPS), emergency power (EP), and normal. The UPS system is located on the third floor electrical closet and is designated for critical systems, such as bioreactors, critical computer systems, and critical analytical equipment. The EP system is designed to come on 10 seconds after a power outage. Critical systems such as cold rooms, refrigerators, freezers, and equipment that can withstand a 10 second outage and still function will be on this system.

Security. The third floor will have a security system from AMAG Access Control Systems, the AMAG 625/675. This system will include electronic proximity detectors for all doors, video surveillance on all corridors, controlled access to the third floor, and an alarm system with motion sensors that are connected to the local police department which will indicate the location and time of unauthorized entrance or motion.

Construction of Plasmids for Expression of Antibodies in CHO Cells

INTRODUCTION

Botulinum neurotoxins (BoNTs) are listed as one of the 6 highest risk threats for bioterrorism by the Centers for Disease Control (CDC) due to their potency, lethality, and ease of production. Botulinum neurotoxin is expressed in the bacteria *Clostridium botulinum* and is one of the most poisonous substances known. The large scale production of humanized monoclonal antibodies (huMabs) with neutralizing activity against toxins (i.e. botulinum neurotoxin) has been identified as one of the highest priorities to counter bioterrorism as drugs for the treatment of botulism are currently unavailable. Recently, potent neutralizing monoclonal antibodies against botulinum neurotoxin serotype A (BoNT/A) were identified, characterized and further cloned to yield humanized Mabs against BoNT/A. It is our goal to develop and optimize a production strategy for the economical large scale production of these antibody based therapeutics for countering BoNT and other agents of biowarfare and bioterrorism. In pursuit of these objectives, using the original plasmid of pcDNA3.1 (+) (Invitrogen) (see Fig. 1) (Selection using G418, Single MCS, CMN promoter) we developed the constriction of plasmids to further produce a humanized monoclonal antibody with neutralizing activity against toxins in the large scale.

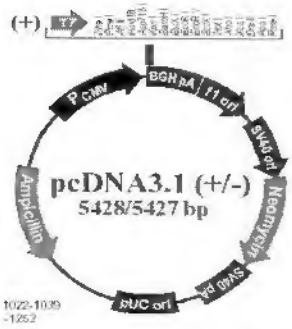

Fig. 1 Plasmid of pcDNA3.1 (+)

EXPERIMENTAL PROCEDURE

Construction of expression vectors

In order to construct plasmids to express humanized monoclonal antibody neutralizing activity against BoNT/A the original plasmids of pcDNA 3.1(+) and pcDNA5/FRT were used and performed the following construction steps as shown at the below. All PCR performed with Hot Star Pfu Turbo (Stratagene) DNA polymerase. All ligations were performed using Quick Ligation kit (Bio-lab laboratories).

1) Construction of plasmid pG1

In order to construct pG1, at first plasmid pcDNA3.1 (+) cut with Pvu II and then relegated. The resulting plasmid were transferred to competent cells of *E. coli* using One Shot- Top10 (Invitrogen) and then, DNA plasmid, isolated from suitable clone.

2) Construction of plasmid pG2

In order to construct pG2, pG1 cut with EcoRV/Hind III, Klenow and relegated. The resulting plasmid were transferred to competent cells of *E. coli* as described above and DNA plasmid, isolated from suitable clone.

3) Construction of plasmid pG3

In order to amplify of the PCR product of fragment β-globin, dhfr, primer pairs were designed on the basis of plasmid ps25. Forward primer contained Mfe1 restriction site and reverse primer contained Nru1 site and the respective sequences of forward and reverse primers used to construct pG4 were

5-C**CAATTG**CGTAGAGCCACACCCTGGTAAG-3 (PS25-βgl-F) and

5-**TTCGCGA**GTTAGTCTTTCTTCTCGTAGACTTC-3 (PS25-βgl-R). PCR was performed with 30 cycles of 95° denaturation for 30 sec, 60°C annealing period of 45 sec., and 72°C extension period 1min, and final extension at 72°C for 10 min, using pS-25 DNA plasmid, as a template. After amplification, the PCR fragment was gel purified, and digested with Mfe1/Nru1 for at last 1h at 37° C and then directionally ligated into plasmid pG2, that was previously digested with both enzymes. Ligated plasmid were transferred into competent cells of *E.coli* as described above and then, DNA plasmid, isolated from suitable clone, sequenced at the Genomics Core Research Facility, University of Nebraska-Lincoln to confirm the sequence.

4) Construction of plasmid pG4

In order to amplify of the PCR product of fragment TKPA, primer pairs were designed on the basis of plasmid PREP4. Forward primer contained Nru1 restriction site and reverse primer contained Mlu1 site and the respective sequences of forward and reverse primers used to construct pG4 were

5-**TCGCGA**GGGGGAGGCTAACTGAAACACG-3 (PREPTK-F) and

5-A**ACGCGT**GCTATGGCAGGGCCTGCC-3 (PREPTK-R). PCR was performed with 30 cycles of 95° denaturation for 30 sec, 60°C annealing period of 45 sec., and 72°C extension period of 1min, and final extension at 72°C for 10 min, using PREP4 DNA plasmid, as a template. After amplification, the PCR fragment were gel purified , and digested with Mlu1/Nru1 for at last 1h at 37° C and then directionally ligated into plasmid pG3, that was previously digested with both enzymes. Ligated plasmid were transferred into competent cells of *E. coli* as described above and then, DNA plasmid, isolated from suitable clone, and sequenced at the Genomics Core Research Facility, University of Nebraska-Lincoln.

5) Construction of plasmid pG5

In order to amplify the PCR product of fragment SV40, MCS-pA primer pairs were designed on the basis of plasmid PREP4. Forward primer contained Bgl II restriction sequence and reverse primer contained Mfe1 sites and the respective sequences of forward and reverse primers used to construct pG5 were

5- A**AGATCT**GGGTACCAGCTGCTAGCAAG-3 (PREPMSV-F) and

5- C**CAATTG**GCGCGAGGCAGCCGGATCATAATC-3 (PREPMSV-R).PCR was performed with 30 cycles of 95° denaturation for 30 sec, 61°C annealing period of 45 sec., and 72°C extension period 1min, and final extension at 72°C for 10 min, using PREP4 DNA plasmid, as a template. After amplification, the PCR fragment were gel purified, and digested with BglII/Mfe1 for at least 1h at 37° C and then directionally ligated into plasmid pG3 that was previously digested with both enzymes. Ligated plasmid were transferred into competent cells of *E.coli* as described above and DNA plasmid, isolated from suitable clone, and sequenced at the Genomics Core Research Facility, University of Nebraska-Lincoln to confirm sequence.

6) Construction of plasmid pG6

In order to amplify the PCR product of fragment CMV primer pairs were designed on the basis of plasmid pcDNA3.1 (+). Both forward and reverse primers contained Bgl II restriction site and the respective sequences of forward and reverse primers used to construct pG6 were 5-**AAGATCT**GTTGACATTGATTATTGACTAG-3 (CMV-F)

5-A**AGATCT**GAGCTCTGCTTATATAGACC-3 (CMV-R). PCR was performed with 30 cycles of 95° denaturation for 30 sec, 51°C annealing period of 45 sec., and 72°C extension period 1min, and final extension at 72°C for 10 min, using PREP4 DNA plasmid, as a template. After amplification, the PCR fragment were gel purified , and digested with Bgl II for at last 1h at 37° C and then directionally ligated into plasmid pG5, that was previously digested with this enzymes. Ligated plasmid were transferred into competent cells of *E. coli* as described above and then, DNA plasmid, isolated from suitable clone, and sequenced at the Genomics Core Research Facility, University of Nebraska-Lincoln to conform the sequence.

7) Construction of plasmid pG7 and pG10

In order to amplify the PCR product of fragment LC primer pairs were designed on the basis of plasmid ps-25. Forward and reverse primer contained Apa1 restriction site and the respective sequences of forward and reverse primers used to construct pG7 and pG10 were 5-ACTA**GGGCCC**ATGAGGGGTCCCCGCTCAGCT-3 (LC-F) and 5-ATCT**GGGCCC**TCAACACTCTCCCCTGTTG-3 (LC-R). PCR was performed with 30 cycles of 95° denaturation for 30 sec, 60°C annealing period of 45 sec., and 72°C extension period 2min, and final extension at 72°C for 10 min, using ps-25 plasmid, as a template. After amplification, the PCR fragment were gel purified, and digested with Apa1 for at least 1h at 37° C and then directionally ligated into plasmid pG5 and pG6 that was previously digested with these enzymes. Ligated plasmid were transferred into competent cells of E.coli and then,

DNA plasmid, isolated from suitable clone, and sequenced at the Genomics Core Research Facility, University of Nebraska-Lincoln to conform the sequence.

8) Construction of plasmids pG8-S25 and pG9-S25

In order to amplify the PCR product of fragment HC primer pairs were designed on the basis of plasmid ps25. Forward primer contained Hind III restriction sequence and reverses primer contained BamH1 sites and the respective sequences of forward and reverse primers used to construct pG8-S25 and pG9-S25 were 5-CTGAT**AAGCTT**CATGGGTTGGAGCCTCATCT-3 (HC-F) and 5-GATCAC**GGATCC**TCATTTACCCGGAGACAGGG-3 (HC-R). PCR was performed with 30 cycles of 95° denaturation for 30 sec, 59°C annealing period of 45 sec., and 72°C extension period 2 min, and final extension at 72°C for 10 min, using ps-25 plasmid, as a template. After amplification, the PCR fragment were gel purified, and digested with Hind3/BamH1 for at least 1h at 37° C and then directionally ligated into plasmid pG7 and pG10 that was previously digested with these enzymes. Ligated plasmid were transferred into competent cells of E. *coli* and then, DNA plasmid, isolated from suitable clone, and sequenced at the Genomics Core Research Facility, University of Nebraska-Lincoln to conform the sequence.

9) Construction of plasmids pG6/GFP and pG9/GFP

In order to amplify the PCR product of fragment GFP primer pairs were designed on the basis of plasmid pS-25-GFP. Forward and reverse primers contained Pci1 restriction site and the respective sequences of forward and reverse primers used to construct pG6/GFP and pG9/GFP were 5-GCTATTC**ACATGT**GAGGTCGAGTGTCAGTCC-3 (SV40GFPzeoSV40-R) and 5-ACTGATT**ACATGT**CTGTGGAATGTGTGTCAGT-3 (SV40GFPzeoSV-F). PCR was performed with 35 cycles of 95° denaturation for 30 sec, a 55°C annealing period of 30 sec., and 72°C extension period 2 min, and final extension at 72°C for 10 min, using pS-25/GFP plasmid, as a template. After amplification, the PCR fragment were gel purified, and digested with Pci1 for at least 1h at 37° C and then directionally ligated into plasmid pG6 and pG9 that was previously digested with this enzyme. Ligated plasmid were transferred into competent cells of E.coli and then, DNA plasmid, isolated from suitable clone, and sequenced at the Genomics Core Research Facility, University of Nebraska-Lincoln to conform the sequence.

10) Construction of plasmid pcDNA5/FRT/HC

In order to amplify the PCR product of fragment HC primer pairs were designed on the basis of plasmid ps25. Forward primer contained *Hind III* restriction sequence and reverse primer contained *BamH1* sites and the respective sequences of forward and reverse primers used to construct pcDNA5/FRT/HC were 5-CTGAT**AAGCTT**CATGGGTTGGAGCCTCATCT (HC-F) and 5-GATCAC**GGATCC**TCATTTACCCGGAGACAGGG (HC-R). PCR was performed with 30 cycles of 95° denaturation for 30 sec, 59°C annealing period of 45 sec., and 72°C extension period 2 min, and final extension at

72°C for 10 min, using ps-25 plasmid, as a template. After amplification, the PCR fragment were gel purified, and digested with *HindIII/BamH1* for at least 1h at 37° C and then directionally ligated into plasmid pcDNA5/FRT that was previously digested with these enzymes. Ligated plasmid were transferred into competent cells of *E. coli* and then, DNA plasmid, isolated from suitable clone, and sequenced at the Genomics Core Research Facility, University of Nebraska-Lincoln to conform sequence.

11) Construction of plasmid pcDNA5/FRT/GFP

In order to amplify the PCR product of fragment GFP primer pairs were designed on the basis of plasmid pTracerEF1α. Forward primer contained Kpn1 restriction sequence and reverse primer contained EcoRV sites and the respective sequences of forward and reverse primers used to construct pcDNA5/FRT/GFP were 5-TA**GGTACC**ATGGCTAGCAAAGGAGAAG-3 and 5-T**AGATATC**TTAATCCATGCCATGTAATCCCAG - 3. PCR was performed with 30 cycles of 95° denaturation for 30 sec, 60°C annealing period of 45 sec., and 72°C extension period 1 min, and final extension at 72°C for 10 min, using plasmid pTracerEF1α, as a template. After amplification, the PCR fragment were gel purified, and digested with *Kpn1/EcoRV* for at least 1h at 37° C and then directionally ligated into plasmid pcDNA5/FRT that was previously digested with Kpn1/EcorV enzymes.Ligated plasmid were transferred into competent cells of *E. coli* and then, DNA plasmid, isolated from suitable clone, and sequenced at the Genomics Core Research Facility, University of Nebraska-Lincoln to conform the sequence.

12) Construction of plasmids pF44/MMF and pF57/MMF

In order to get the MMF fragment, oligo1 and oligo 2 primers were used. Oligo 1 primer contained *Mlu1* restriction site and Oligo2 primer contained *Mfe1* restriction site. Oligo1 and Oligo 2 primers at first heated to 95°C and then slowly cooled down to room temperature. The sequences of Oligo 1 and 2 were, 5-ACACGCGTGCATCGATGACCTAGGTACAATTGGA-3 and 5-TGTGCGCACGTAGCTACTGGATCCATGTTAACCT-3, respectively. After getting MMF fragment, digested with *Mlu1/Mfe1* for at least 1 hour at 37°C, purified with PCR purification kit (QIAGEN,) and then directionally ligated into plasmids PcDNA/FRT-F44 and PcDNA/FRT-F57, that was previously digested with *Mlu1/Mfe1* enzymes. Ligated plasmid were transferred into competent cell of *E. coli* and then, DNA plasmids, isolated from suitable clones sequenced at the Genomics Core Research Facility, University of Nebraska-Lincoln to conformed the sequence.

13) Construction of plasmids pF44/MMF/GFP and pF57/MMF/GFP

In order to amplify the PCR product of fragment GFP primer pairs were designed on the basis of plasmid pTracerEF1α. Forward primer contained *Kpn1* restriction sequence and reverse primer contained *EcoRV* sites and the respective sequences of forward and reverse were 5-TA**GGTACC**ATGGCTAGCAAAGGAGAAG-3 and

5- TA**GATATC**TTAATCCATGCCATGTAATCCCAG-3 PCR was performed with 30 cycles of 95° denaturation for 30 sec, 60°C annealing period of 45 sec., and 72°C extension period 1 min, and final extension at 72°C for 10 min, using plasmid pTracerEF1α, as a template. After amplification, the PCR fragment were gel purified, and digested with *Kpn1 EcoRV* for at least 1h at 37° C and then directionally ligated into plasmid pF44/MMF and pF57/MMF that was previously digested with *Kpn1 EcoRV* enzymes. Ligated plasmids were transferred into competent cells of *E coli* and then, DNA plasmids, isolated from suitable clones, sequenced at the Genomics Core Research Facility, University of Nebraska-Lincoln to conform the sequence.

14) Construction of plasmids pF44/MMF/HC and pF57/MMF/HC

In order to amplify the PCR product of fragment HC primer pairs were designed on the basis of plasmid ps25 Forward primer contained *Hind III* restriction sequence and reverse primer contained *BamH1* sites and the respective sequences of forward and reverse were 5-CTGA**TAAGCTT**CATGGGTTGGAGCCTCATCT-3 (HC-F) and 5-GATCAC**GGATCC**TCATTTACCCGGAGACAGGG-3 (HC-R). PCR was performed with 30 cycles of 95° denaturation for 30 sec, 59°C annealing period of 45 sec., and 72°C extension period 2 min, and final extension at 72°C for 10 min, using ps-25 plasmid, as a template. After amplification, the PCR fragment were gel purified, and digested with *Hind III BamH1* for at least 1h at 37° C and then directionally ligated into plasmids pF44/MMF and pF57/MMF that was previously digested with these enzymes.

Ligated plasmids were transferred into competent cells of E. *coli* and then, DNA plasmids, isolated from suitable clones, and sequenced at the Genomics Core Research Facility, University of Nebraska-Lincoln to confirm the sequence

Cell Line, Media, Transfection and Expression Vectors

The CHO-DG44 cell line, obtained from Dr. Larry Chasin of Columbia University, was used a host because it is dhfr negative and can be amplified for gene expression upon methotrexate addition. The host cell line was maintained in α-MEM media (Invitrogen) supplemented with 8% fetal bovine serum (FBS) (Invitrogen). The cells were transfected with plasmid pG9-S25 which I have constructed. (See Fig 5) The pG9-S25 plasmid was constructed by inserting the light and heavy chain IgG genes against BoNT serotype A, along with the gene for dhfr into the plasmid pG6 (Figure 4) The CHO-DG44 cell line was transfected with the pG9-S25 plasmid using Lipofectamine 2000 (Invitrogen) Briefly, cells were seeded with 0 5 ml in 24 cell plates at 2 x 10^5 cell ml in α-MEM media with 8% FBS and grown overnight. 1 μg plasmid DNA and 0.5-5.0 μl Lipofectamine 2000 were combined in 0.1 ml Opti-MEM media (Invitrogen) and allowed to equilibrate for 20 min. 0.5 to 2 ml of Lipofectamine 2000 proved to be optimal. Plasmid DNA was added to the transfection mix either uncut or linearized with SspI. This site was chosen since it would place the amplifiable gene (dhfr) between the heavy and the light chains. The DNA/Lipofectamine 2000 solution was added to the 24 well plates and the plates were incubated at 37°C overnight. Stably transfected cells were selected in α-MEM media lacking ribonucleotides and deoxyribonucleotides α-MEM (-) and with 8% FBS, which prevents cells lacking dhfr from growing. Cells were passaged several times and

individual clones were obtained by dilution cloning at 0.5 cells/well in 96 well plates. Samples were taken from wells containing growing cells after 16-18 days. The antibody concentrations were determined using an ELISA specific for the Fc portion of a human heavy chain of IgG (Figure 8).

ELISA

The concentration of the whole antibody, as well as the concentration of the light and heavy chain portions were determines by an enzyme-linked immunosorbent assay. Affinity purified rabbit anti-human IgG antibodies were diluted to 5 µg/m. in coating buffer consisting of 100 mM $NaHCO_3$ and 100 mM NaCl (pH = 9.3) 100 µl diluted antibody was added to 96 well plates (Nunc) and incubated overnight at 4°C The plates were washed twice in Tris buffer (20 mM Tris-HCl, 50 mM NaCl, pH = 7.2) containing 0.1% Tween 20 and then twice in Tris buffer alone Blocking buffer (Tris buffer with 0.5% BSA) was added to the 96 well plates and incubated at 37°C for 1 h Supernatant samples were diluted in the blocking buffer and samples were loaded into the 96 well plates in triplicate. Plates were incubated for 1 h at 37°C and the washing procedure was repeated. 100 µl of a goat anti-human IgG-HRP conjugate antibody diluted to 0.5 to 2 mg/ml in the dilution buffer was added to the plates The plates were incubated for 1 h at 37°C and the washing procedure was repeated. Lastly, 100 ml of 50 µg/ml ABTS in ABTS buffer (Roche) was added to the plates. The absorbance was determined at 405 nm using a ELx800 plate reader (Bio-Tek). This procedure was used for whole antibody, heavy chain (Fc specific), and light chain (κ specific). Whole, Fc and kappa rabbit anti-human IgG coating antibodies and whole, Fc and kappa goat anti-human IgG-HRP conjugated antibodies were used in the Eliza's (Sigma).

Western blotting

Supernatant samples from growing cultures were obtained by centrifugation at 1200 rpm for 5 min Western blot was performed using NuPAGE (Invitrogen) according to manufacture's protocol on 10 % acrylamide gels, and gels were subsequently blotted to PVDF membranes using 1xNuPAGE Transfer Buffer (Invitrogen) at 30 V for 1 hour. Blots were blocked with 5% nonfat dry milk in TBS buffer for 2 h at room temperature Recombinant humanized monoclonal antibody was detected by incubating with Anti-Human IgG kappa specific and Goat Anti-Human IgG Fc-specific (Sigma) in 5% nonfat dry milk in TBS buffer for 1 h at room temperature. The protein bands were detected using ECL kit (Amersham). Human IgG (Sigma) was used as a positive control. For non-reducing gels, the loading buffer lacked SDS and β-mercaptoethanol

Transfer to Suspension Culture

After screening the clones for antibody production, all clones that reached 100ng/ml antibody after three days were transferred to suspension culture These clonal cell lines were frozen and can be analyzed for antibody production along with the methotrexate gene amplified cell lines, once the methotrexate resistant clones are transferred to suspension culture. To transfer cells to suspension culture, the cells were grown in 50 ml spinner flasks (Wheaton)

with decreasing concentration of FBS. Initially the cells were seeded in the spinner flasks at 2-3 x 10^5 viable cells/ml in CHO-S-SFMII media (Invitrogen) containing 1% FBS, and were passaged every 2-4 days into fresh media containing decreasing amount of FBS. After 8-10 passages the cells were frozen in α-MEM media with 10% FBS and 10% dimethylsulfoxide (DMSO) (Fisher) at a cell density of 10^7 cell/ml. The cells were slowly frozen at -80°C in 1.5 ml aliquots and were stored in liquid nitrogen for later use.

Growth of Cells in Suspension Culture

The CHO-DG44 pG9-S25 cells were transferred to suspension culture in IS-CHO-V (Irvine Scientific) media by slowly decreased FBS content in spinner flasks. Cells were initially seed at 3 x 10^5 cells/ml in 50 ml IS-CHO-V with 1 % FBS. Cells were fed every 2-4 days with slowly decreasing levels of FBS. Cells were frozen and stored in liquid nitrogen after approximately 10-12 passages. For the batch runs, cells were taken for frozen stock and allowed to grow fro 3-5 passages and were the seeded in IS-CHO-V media at 2.2 x 10^5 cells/ml in IS-CHO-V media. Cell counts were determined using a hemacytometer and S25 antibody production was analyzed by ELISA.

RESULTS

1. CONSTRUCTION OF PLASMIDS

In order to get the expression plasmids for producing a humanized monoclonal antibody neutralizing activity against BoNT/A in the large scales the following plasmids has been constructed:

1	PcDNA3.1 (+)/Neo (-) (pG1)	Cut pcDNA 3.1(+) with Pvu II and removed PvuII fragment from pcDNA3.1 (+) and relegate
2	PcDNA3.1 (+)/Neo (-)/MCS$_1$ (pG2)	Cut pG1 with Hind III and EcoR V, Klenov and truncate MCS in 1, relegate
3	PcDNA3.1 (+)/Neo (-)/MCS$_1$/β-globin.dhfr (pG3) **(See Fig. 2)**	PCR amplification of fragment β-globin .dhfr from plasmid ps25 and cut plasmid pG2 with Mfe I and Nru I and inserted β-globin, dhfr into this plasmid in front of CMV promoter
4	pG3/TKpA (pG4)	PCR amplification of fragment TKPA from PREP4- and cut of pG3 with *Nru I* and *Mlu I* and insert TKpA after dhfr into this plasmid
5	pG3/TKpA/MCS.SV40pA (pG5) **(See Fig. 3)**	PCR amplification of fragment of SV40, MCS From PREP4 and cut pG4 with *Bgl II* and *Mfe I* and inserted SV40, MCS fragment into this plasmid, upstream of β-globin promoter in pG4
6	pG3/TKpA/MCS.SV40pA/CMV (pG6) **(See Fig. 4)**	PCR amplification of fragment CMV from pcDNA 3.1(+) and cut pG5 with *Bgl II* and inserted this CMV fragment in front of MCS2 in plasmid pG5
7	pG5/S25.LC (pG7)	PCR amplification of LC from ps25 plasmid and cut pG5/S25 with *Apa1* and inserted LC fragment in MCS1 of pG5.
8	pG5/S25.LC/S25.HC (pG8)	PCR amplification of HC from ps25 plasmid And cut pG5/S25/LC with *BamH1/HindIII* and inserted HC fragment in MCS2 of pG7.
9	pG6/S25/LC (pG10)	PCR amplification of LC from ps25 plasmid and cut pG6/S25 with Apa1and inserted LC fragment into pG6.
10	pG6/S25 LC/S25.HC (pG9-S25) **(See Fig. 5)**	PCR amplification of HC from ps25 plasmid and cut pG10 with *BamH1/HindIII* and inserted HC Fragment into pG10 plasmid.
11.	pcDNA5/FRT/HC (see,Fig,6)	PCR amplification of HC from ps25 plasmid and cut pCDNA5/FRT with *Bam HI* and *Hind III* and inserted HC into this plasmid in the MCS of pc DNA 5/FRT
12	pcDNA5/FRT GFP	PCR amplification of GFP from pTracerEF1α and Cut pc DNA5/FRT with Kpn *I* and *EcoRV* and Inserted GFP into this plasmid.
13	pF44/MMF	Cut PcDNA5/FRT- F44 plasmid with *Mlu I/Mfe* and truncate and then *MluI / Mfe I* cut MMF-fragment ligate into this plasmid
14	pF57/MMF	Cut PcDNA5/FRT- F57 plasmid with *Mlu I/Mfe* and truncate and then *MluI/Mfe I* cut MMF- fragment ligate into this plasmid
15	pF44/MMF/GFP	PCR amplification of GFP from pTracerEF1α and cut pF44/MMF with *Kpn1/EcoRV* and inserted GFP into pF44/MMF.
16	pF57/MMF/HC	PCR amplification of HC from ps25 plasmid and cut pF57/MMF with *HindIII BamH I* and inserted HC into pF57/MMF.
17	pF57/MMF/GFP	PCR amplification of GFP from pTracerEF1α and cut pF57/MMF with *Kpn1/EcoRV* and Inserted GFP into pF57/MMF.
18	pF44/MMF/HC	PCR amplification of HC from ps25 plasmid and cut pF44/MMF with *HindIII BamH1* and Inserted HC into pF44/MMF.

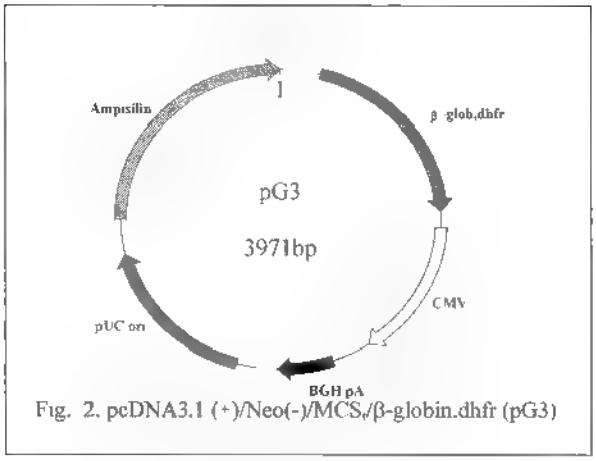

Fig. 2. pcDNA3.1 (+)/Neo(-)/MCS,/β-globin.dhfr (pG3)

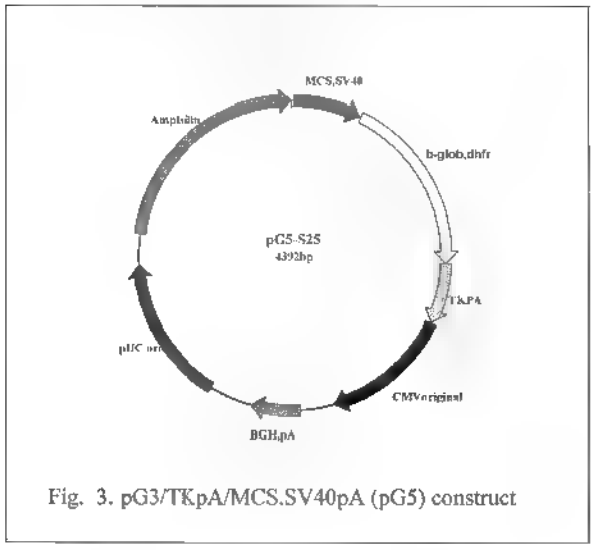

Fig. 3. pG3/TKpA/MCS.SV40pA (pG5) construct

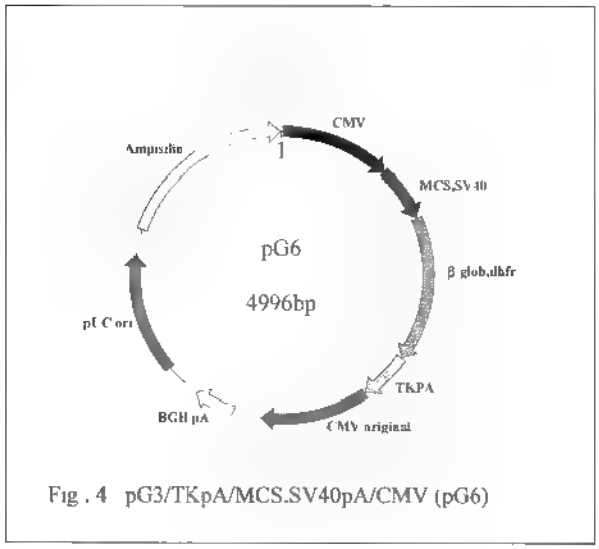

Fig . 4 pG3/TKpA/MCS.SV40pA/CMV (pG6)

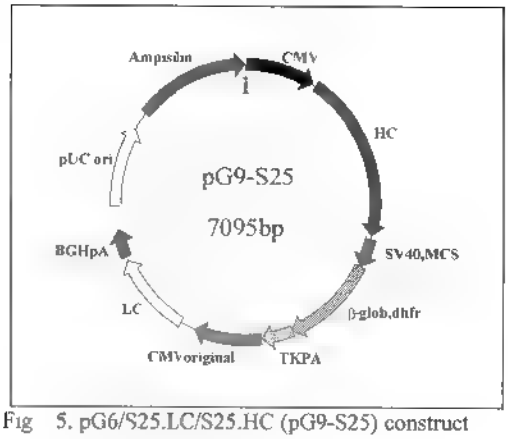

Fig 5. pG6/S25.LC/S25.HC (pG9-S25) construct

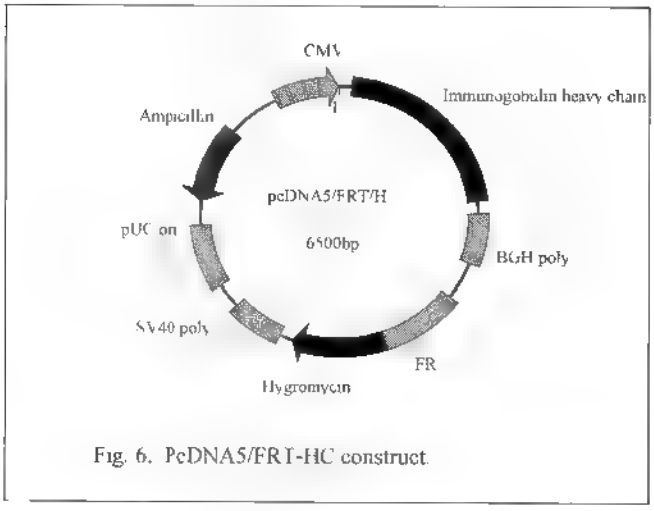

Fig. 6. PcDNA5/FRT-HC construct

Construction of GFP (Green Fluorescence Protein) Plasmids

To decrease the time and effort required to isolate high expressing gene amplified cell lines, I constructed the plasmid pG6-GFP and pG9-GFP (see Fig. 6 and 7), containing GFP-zeocin.

PG6-GFP.zeo. (pG3/TKpA/MCS SV40pA CMV) (see Fig 6). It took 6 steps (see at the above) to construct of pG6. This plasmid contains two CMV promoters and two different multiple cloning sites for the insertion of the light and heavy chain. In between these it contains dhfr driven by a β- globin promoter and 3 different polyadenylation sites. After inserting the GFP to this plasmid, we are hoping that this will allow for greater plasmid stability and will be an important plasmid for the insertion of the light and heavy chain from any antibody into a single plasmid.

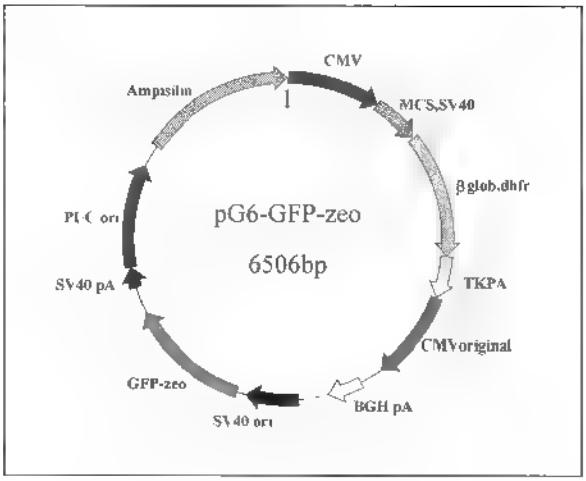

Fig. 7. pG6-GFP-zeo. construct

pG9/ GFP.zeo. pG9 is the plasmid (see at the above) and constructed after insertion of the light and heavy chain to the pG6. After insertion of GFP to this plasmid we expecting that this will be giving very high antibody production upon selection in both Zeocin and α-MEM (-) This plasmid will allow for screening of amplified cells by flow cytometry, providing us the ability to analyze a significantly higher number of cell lines. This also gives us the ability to concentrate cells with high GFP production during the gene amplification process. Once again, cells with high GFP content will likely have high antibody productivities The production of GFP and S25 antibody will be established by transfecting CHO-DG44 cells and generating stable clones

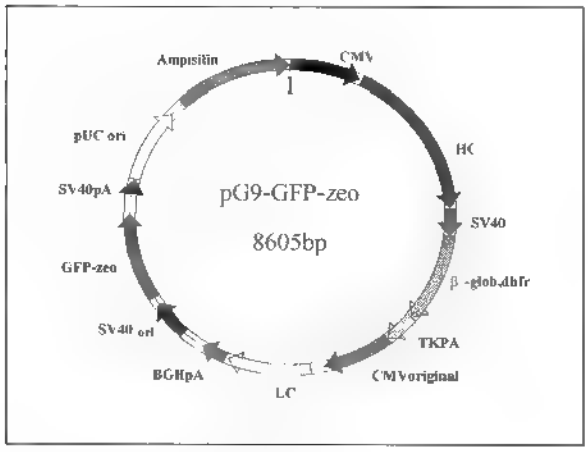

Fig.8. pG9-GFP-zeo. Construct

2. TRASFECTION, ELISA and WESTERN BLOTTING

Samples were taken from wells containing growing cells after 16-18 days. The antibody concentrations were determined using an ELISA specific for the Fc portion of a human heavy chain of IgG (Figure 9). Several clones that showed antibody titers greater than 100 ng/ml were selected for further Western analysis. Presence of the light and heavy chain was confirmed by Western blotting (Figure 10, 11). Cells with an antibody concentration of greater than 100 ng/ml were transferred first to 24 well plates and then to 25 cm^2 T-flasks Cells were seeded at 2 x 10^5 cells/ml in 9 ml α-MEM (-) supplemented with 8% FBS in a 25 cm^2 T-flask Clone #13 showed expression levels greater than 100ng/ml after three days in adherent cultures in the 25 cm^2 T-flasks transferred to 75-cm^2 T-flasks and 1x10^7 cell/ml cells frozen in liquid nitrogen until use.

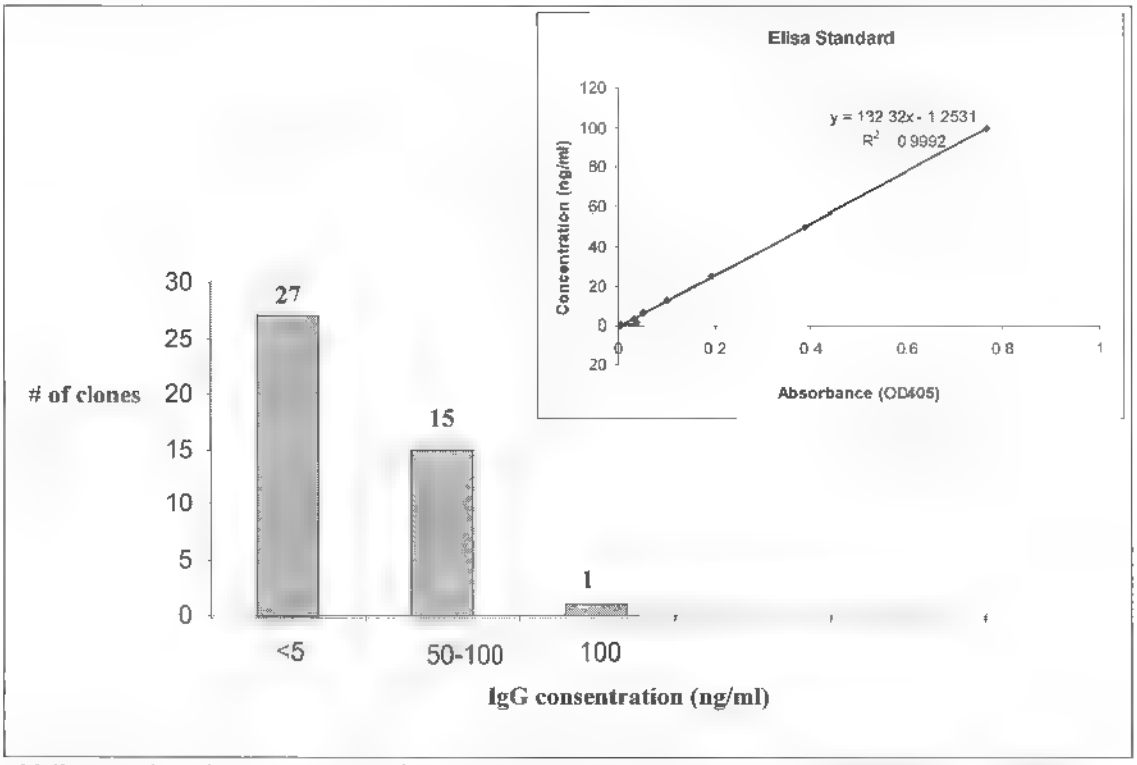

Fig. 9. Initially screening of CHO-DG44 transfectants (cells trasfected with pG9-S25 plasmid) using an Fc specific ELISA. Notable, one clone (#13), had antibody content greater than 100 ng/ml.

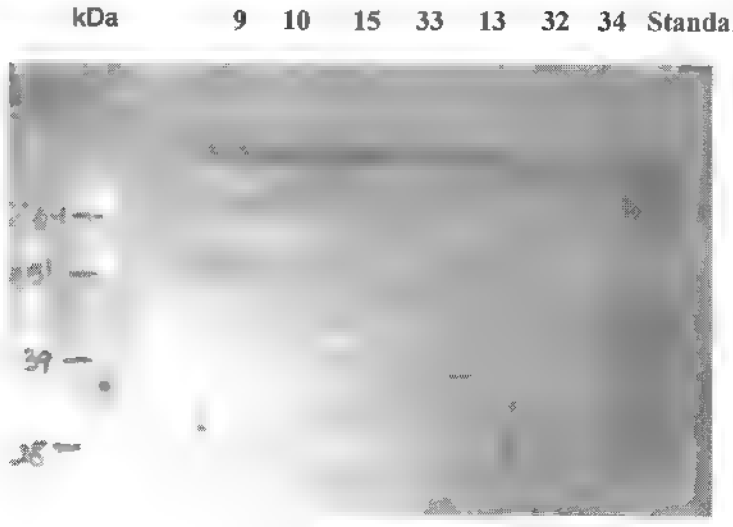

Fig. 10. Western blot analysis of the clones using Goat Anti-Human IgG Fc-

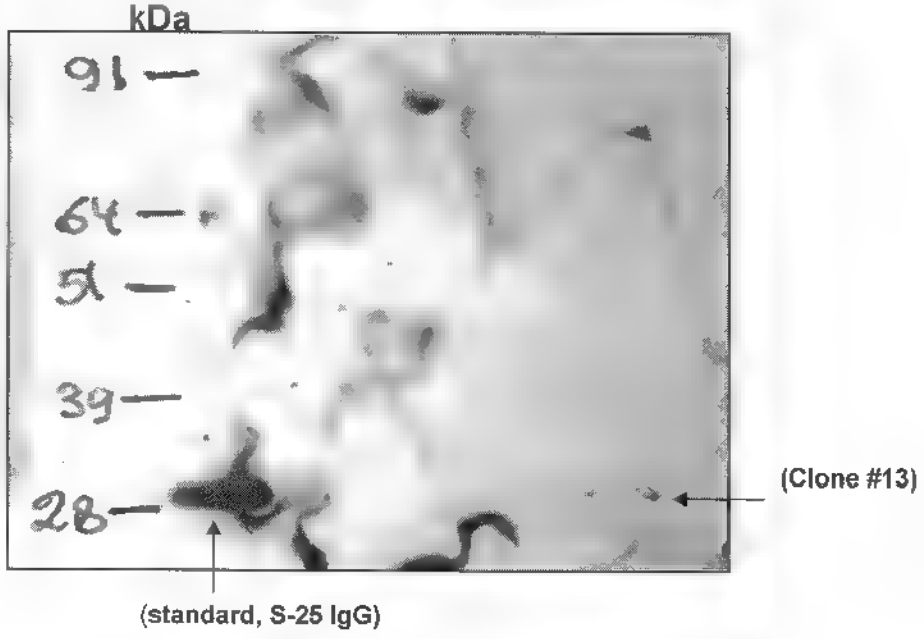

Fig. 11. Western blot analysis of the clone #13 using Anti-Human IgG kappa

CO-TRANSFECTION WITH PLASMIDS pcDNA5/FRT-HC and POG44

The cell line Flp-In CHO was co-trasfected with plasmids pcDNA5/FRT-HC and POG44. The host cell line was maintained in α-MEM (+) media (Invitrogen) supplemented with 8% Fetal bovine serum (FBS) (Invitrogen) Transfection was performed as described above. After trasfection, the selection was performed in 400 µg ml hydromaycin. The antibody concentrations were determined using an ELISA specific for the Fc portion of a human heavy chain of IgG (Figure 12). We found that several clones possess antibody productivity and one clone #2 had about 1000 ng ml antibody concentration. The clones that had antibody productivity analyzed by western blotting using IgG anti-Human Fc-specific (Figure 13). The purpose of this co-trasfection was as positive control. In near future we are planning to insert of active elements of CMV promoter to this pcDNA5/FRT-HC plasmid. We will check how these promoter elements affect of production of heavy chain.

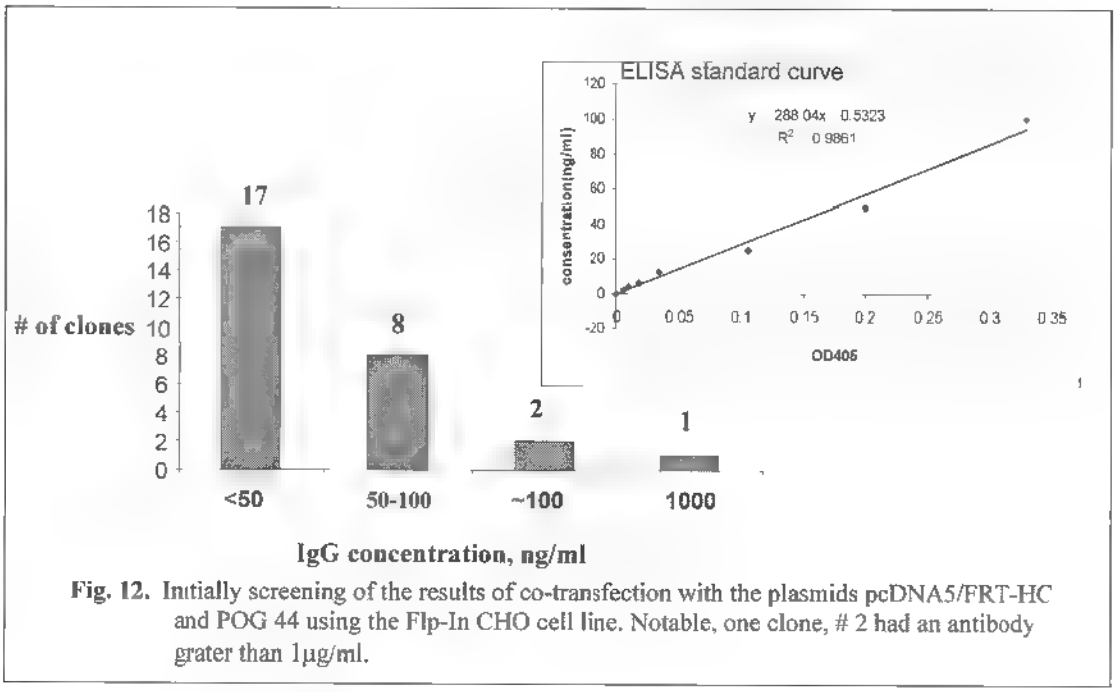

Fig. 12. Initially screening of the results of co-transfection with the plasmids pcDNA5/FRT-HC and POG 44 using the Flp-In CHO cell line. Notable, one clone, # 2 had an antibody grater than 1µg/ml.

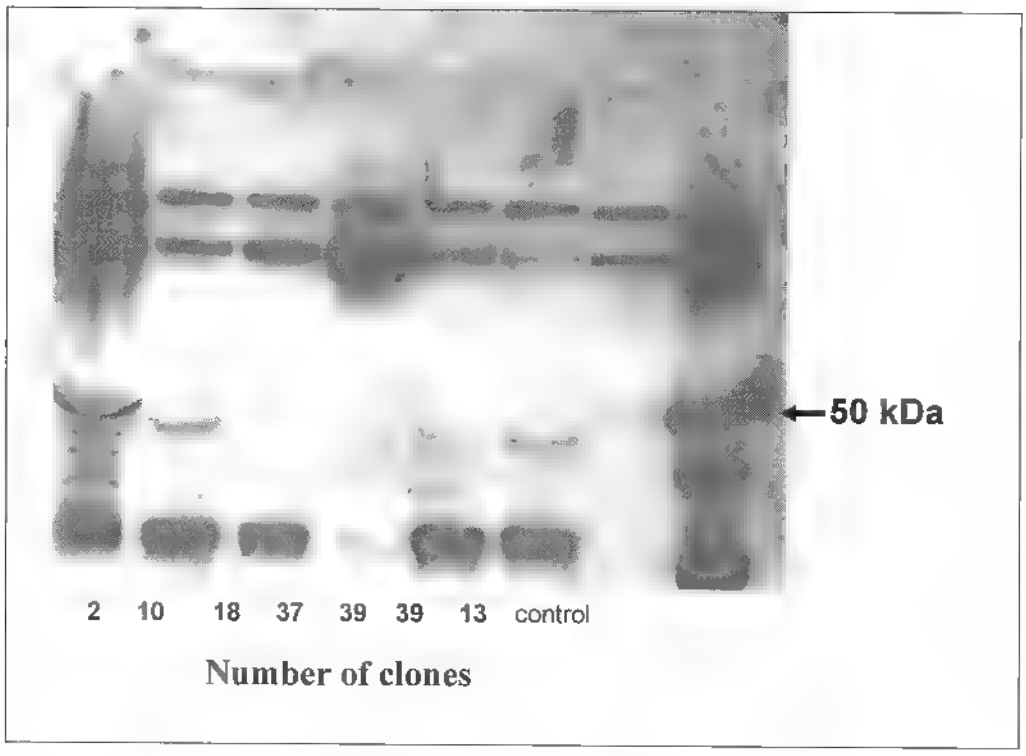

Fig. 13. Western blot analysis of the clones using IgG anti-Human Fc-specific.

Development of serum-free media in CHO-DG44 cells using a central composite statistical design.

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Abstract:

A serum free media was developed for the production of recombinant antibody against Botulinum Neurotoxin A (BoNTA) designated as S25, using dihydrofolate reductase deficient Chinese Hamster Ovary Cells (CHO-DG44) in suspension culture. An initial control basal medium was prepared, which was similar in composition to HAM'SF12 IMDM (1:1) supplemented with insulin, transeferrin, selenium and a lipid mixture. The vitamin concentration of the basal medium was twice that of HAM'SF12 IMDM (1:1). CHO-DG44 cells expressing S25 antibody grew from 2×10^5 cells to maximum cell density of 1.04×10^6 cells/ml after five days in this control media. A central composite design, which is the most popular of the many classes of response surface methodology designs (RSM), was used to identify optimal levels and interaction among five groups of media components glutamine, Essential Amino Acids (EAA) Non Essential Amino Acids (NEAA), Insulin Transferrin Selenium (ITS) and lipids. Fifty experiments were performed out in four batches, with two controls in each batch. There was little effect of ITS and lipid concentrations over the range studied, and glutamine concentration showed a strong interaction with EAA. The optimal concentration of the variables studied were 2.5 mM Glutamine, 7.4 mM (3.25X) EAA, 1.4 mM (0.5X) NEAA, 1X ITS supplement, 0.7X Lipids supplement. The maximum viable cell density attained in the optimized media was 1.4×10^6 cells/ml, a 35% improvement over the control culture, while the final antibody titer attained was 22 ± 3.4 µg/ml, a 50% improvement.

Introduction:

Botulinum neurotoxins produced by the anaerobic Clostridium botulinum, is one of the most potent toxin known to humans (1, 2). There are seven serologically different botulinum toxins (BoNT A to G), which are highly potent protein toxins that inhibit neurotransmitter release from peripheral cholinergic synapses. Among the seven types, BoNT A, B, E, F cause human disease botulism. The active neurotoxin is synthesized as two polypeptide chains, a heavy chain (100KDa) and light chain (50KDa) connected via disulfide linkage (3,4). Recombinant monoclonal antibodies (mAb) can neutralize the effects of BoNT with out requiring human donors for plasmapheresis (5). Recently, potent neutralizing monoclonal antibodies were identified, characterized, cloned and expressed in Chinese Hamster Ovary (CHO) cells (6).

The large-scale, commercial production of therapeutically important proteins from rCHO cells typically involves a suspension culture-based manufacturing process (7).

It is desirable to use serum-free media in suspension culture because serum can cause problems in subsequent processes (8, 9). However, there is no universal serum-free media applicable to all cell lines. There is need to develop specific media suitable for each cell line (10, 11).

The media used for an animal cell culture are very complex. Because the significance of particular compounds for cell growth or product synthesis is difficult to fully understand, statistical methods are adopted to develop animal cell culture media. The traditional one-factor-at-a-time approach, which is generally used at starting stages of optimization where most important factors can be identified eliminating the insignificant ones, is time consuming and is incapable of reaching the true optimum because of interactions among the factors under study.

Moreover, this approach assumes that the various growth parameters do not interact and the process response is a direct function of a single varied parameter (12, 13). In reality, the observed behavior of growth results from the interactive influences of the various variables (14) To be effective, optimization requires, statistical methods that take these interactions in to account.

Response surface methodology (RSM), an experimental strategy for seeking the optimum conditions for a multivariable system, is a much more efficient technique for optimization (15) RSM comprises mathematical and statistical procedures that can be used to study relationships between one or more responses and a number of independent variables. In addition to analyzing the effects of independent variables, this experimental methodology generates a mathematical model that accurately describes the overall process (16, 17). RSM has been employed to solve the multivariate problems and optimize responses in many types of experimentation (18, 19, 20). In this approach, concentrations of medium components are the variables; each variable refers to some base value and varies in a certain pattern This pattern is designed by using statistical methods to yield the most information by a minimum number of experiments.

In this study we adopt an RSM approach to locate the optimum levels of Glutamine, Essential Amino Acids (EAA), Non Essential Amino Acids (NEAA), Insulin, Selenium, Transferrin (ITS), and Lipids. Our objective is to gain insight in to the interactions among these factors that significantly impact the viable cell density. Glutamine acts as the primary source of nitrogen as well as additional carbon and energy source. It contributes precursors to the formation of the major intracellular binding blocks: amino acids, proteins. Approximately 30 to 65% of the cell energy requirement is derived from glutamine metabolism (21, 22). Amino acids are primary sources of nitrogen that protect cells from nutrient deprivation (23), elevated osmolarity (24) and elevated pCO_2 (25). Insulin serves as a growth and maintenance factor for cells and is considered to be important for serum free cultures (26). Insulin stimulates uridine and glucose uptake, and synthesis of RNA, protein and lipid, it also increases fatty acid and glycogen synthesis (27). Transferrin is one of the most essential growth promoting supplements in serum-free media, and its omission causes severe inhibition of cell growth (28). Transferrin is an iron binding glycoprotein that interacts with surface receptors. It is closely related to the transport of iron across the plasma membrane (29). Transferrin has additional in vitro functions e g., chelation of deleterious trace materials, that are unlikely replaced by other components. Selenium is a trace element essential for mammalian cell cultures (30) mechanism is poorly understood There is evidence that selenium enhances growth rate in serum-free cultures (31). Lipids are required for proliferation, differentiation, and antibody secretion. They play a major role in the cell membrane, which is composed of a phospo-lipid bi-layer, and help in the transmission of nutrients into the cell and excretion of proteins out the cell (32) The major important functions exhibited by the variables motivated us to choose the response surface methodology in order to observe the higher order interactions that would maximize the response.

Results were analyzed statistically by SAS program and optimum conditions were selected graphically. Interactions among these factors were also examined.

Materials and Methods

Cell Line and Media:

The parental cell line was obtained by weaning CHO-DG44 from the serum according to standard cell culture techniques (33). The base medium used during the weaning process was a commercial serum-free medium CHO-S-SFMII, known to contain animal-derived proteins and hydrolysates. The process of weaning CHO-DG44 of its serum dependence lasted approximately 4-5 months. The resulting cell line was used as the starting point for all subsequent development efforts including recombinant cell line generation, medium development studies Recombinant cell lines were derived from our parent cell .ine using standard molecular biology techniques

Media:

The basal medium was prepared similar to the HAM'S: IMDM (1:1) media excluding Hypoxanthine and Thymidine, by adding components separately. The concentration of the inorganic salts, and other components such as linoleic acid, lipoic acid, phenol red, putrescine 2HCl, sodium pyruvate, and HEPES is same as HAM'SF12.IMDM (1.1). The concentrations of the glucose and glutamine in the starting basal media were 4 g/L, and 4mM. For amino acids the media was supplemented with 1.75 X of EAA and 1.75X of NEAA, which come from Gibco as 50X and 100X solutions respectively. The concentrations of individual amino acids in EAA (50X) and NEAA (100X) solutions are given in Table 1. The additional components added to the basal media are vitamins the concentrations of which were double those in HAM'SF12:IMDM (1 1), 1X ITS, and 0.7 X Lipids Supplement, which come as 100X solutions from Gibco. The concentration of the Individual components in ITS and Lipids Supplements are given in the Table 1. The composition of the complete basal media is given in Table 1(a).

Cell culture·

The cell cultivation was performed in 37°C humidified incubators supplemented with 5% carbon dioxide. The seeding density was 2×10^5 cells/ml, and cell counts were performed every four days. The number of cells was determined using a hemocytometer. Spheroids would be enzymatically dissociated when spherical aggregates were observed. Two milliliters (ml) of sample were harvested from spinners and placed in a 1.7 ml microtube and centrifuged at 1200 rpm for six minutes; 1.5 ml supernatant was saved for antibody assays. 100 µl of trypsin solution (2.5% (w/v) Trypsin in PBS) was added to resuspend the cells of 400 µl. The cells were incubated at room temperature for f fteen minutes and the cell density and viability were then determined by the trypan blue exclusion method.

Antibody Assay:

Antibody concentration was determined using ELISA. Affinity purified rabbit anti-human IgG antibody was diluted to 5 µg ml in coating buffer (100 mM $NaHCO_3$ and 100 mM NaCl (pH – 9.3)) and volume of 100 µl was added to the wells of 96 well plates and incubated overnight at 4°C. The plates were washed twice in Tris buffer (20 mM Tris-HCl, 50 mM NaCl, pH – 7.2) containing 0.1% Tween 20 and then twice in Tris buffer alone Blocking buffer (Tris buffer with 0.5% BSA) was added to the 96 well plates and incubated at 37°C for 1 h Supernatant samples were diluted in the blocking buffer and samples were loaded into the 96 well plates in triplicate.

Plates were incubated for 1 h at 37°C and the same washing procedure was repeated. 100 µl of a goat anti-human IgG-HRP conjugate antibody diluted to 0 5 to 2 mg ml in the dilution buffer was added to the plates The plates were incubated for 1 h at 37°C and the washing procedure was repeated. Lastly, 100 µl of 50 µg ml ABTS in ABTS buffer (Roche) was added to the plates. The absorbance was determined at 405 nm using an ELx800 plate reader (Bio-Tek).

Experimental Design

Response surface methodology (RSM) was used to determine the influence of some medium components on the response of viable cell density. Our assumption is that since our product is growth-associated, increase in viable cell density will ultimately increase the antibody production. Theoretical and fundamental aspects of RSM have been extensively discussed else where (16). The experimental design adopted Box's central composite design for five variables at five levels each. The five independent variables were X1 - Glutamine, X2 EAA, X3 NEAA, X4 = ITS, X5 – Lipids. The independent variable coded regions were -α (-2, Lowest Level), -1, 0 (middle level), 1, and +α (2, highest level) The actual values, which were chosen from preliminary studies, and the corresponding coded and uncoded values of the five independent variables are given in Table 2. The complete design has 42 experimental points, which including 8 replications of the center point. The treatment combinations and observed responses are presented Table 3. The 50 experimental media runs were prepared in random order and the experiments were performed in four batches. The dependent variable (Y) was viable cell density and was assumed to be affected by the five independent variables. Based on data from this design we fit a second order or higher order polynomial regression model described as follows:

$$Y = b_l + \sum_{i=1}^{k} biXi + \sum_{i=1}^{k} b_{ii}X_i^2 + \sum_{i}\sum_{j} bijXiXj + \epsilon$$

where

Y – Viable Cell density

b_l Intercept for block l

X – Factors (X1 - Glutamine, X2 – EAA, X3 – NEAA, X4 = ITS, X5 – Lipids)

b_z = regression coefficient ($z = i$, ii or ij, *where* $i<j$)

ϵ – Residual Error

k = 1,2,3.....

Statistical analysis.

Using ordinary least squares the regression model was fit to evaluate, the explanatory variables regarding linear, interaction, and quadratic effects of coded levels of Glutamine, EAA, NEAA, ITS, Lipids on cell density. The R^2 value was used to evaluate model sufficiency and the α-level was set as 5 percent at which point every term in the selected model should be significant. The reduced model was evaluated using the R^2 .Lack of fit and was used to

attempt to find optimal conditions for all the variables maximizing the cell density Canonical analysis was then used to evaluate the nature of the stationary point (maximum, minimum or saddle) and to find the ridge of steepest ascent. Further experiments were carried out in the direction of the maximum response along with alternate experiments where Glutamine was set to different coded levels from '0' to '-3', keeping EAA constant at coded level '2' and '4'. All statistical computations were done using SAS/STAT procedures, and optimum conditions were found through SAS data-step programming. Response surface plots were generated by SAS/GRAPH

Results and Discussion:

Regression analysis revealed that linear and quadratic effects were more significant than cross product interactions (Table 4) Among all independent variables, Glutamine (negative effect) and NEAA (positive effect) had the greatest effects on the cell density, while EAA showed an effect when combined with Glutamine. Among the pair wise interactions EAA and Glutamine exhibited the greatest effect. Although NEAA squared and NEAA by itself were significant they did not have a great effcet when compared with the other variables. The response surface plots are plotted to see the effect of EAA and NEAA (Fig 1), EAA and Glutamine (Fig 2), NEAA and Glutamine (Fig 3) on the response which is viable cell density (Y). ITS and Lipids were found to have no effect. The R^2 value for the total model is 0.6339 To simplify the model, the variables ITS and Lipids were removed from the model and the data were re-analyzed using the reduced model. The polynomial regression model used for three variables was

$$Y = b_o + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_{11}^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + e$$

When the variables ITS and Lipids kept constant, the lack of fit is insignificant suggesting that the model is adequate to explain the effect of these three variables on the response. The R^2 value of the reduced model decreases to 0 502 As Table 5 shows, the Glutamine had significant linear effects and interacted with EAA. EAA and NEAA had significant quadratic effects while NEAA also had a linear effect on the cell density.

Canonical Analysis:

Canonical analysis is a mathematical approach used to examine the overall shape of the response surface and to determine if the estimated response point is a maximum, minimum or a saddle point. If the stationary point is maximum or minimum, a corresponding increase or decrease will result in the response. In case of a saddle point, the response may increase or decrease when we move away from the stationary point, depending on which direction is taken. Maximizing the viable cell density is of interest; however the stationary point was a saddle point, so we move on the ridge in the direction towards attaining the maximum response.

The points on the ridge that increased the response were found using the RIDGEMAX option of the SAS/RSREG procedure, and are shown in Table 6.

From the Table 5 and 6, glutamine showed a negative effect on cell density while EAA, NEAA showed a positive effect. Therefore the Glutamine values for the ridge moved in the negative direction and the values for EAA and NEAA moved in the positive direction. Following the ridge in Table 6 the highest cell density was 1.37E+06 cells/ml, but this prediction was not very reliable due to a large standard error. Based on the ridge analysis, the Glutamine with high cell densities were decreasing below a level of 1mM. Glutamine values smaller than 1mM were

thought to be unreasonable and therefore additional experiments should be conducted on the ridge below Glutamine values of 1mM.

To further explore the surface, we used the reduced model from the Table 6, and obtained predicted cell densities with a Glutamine constant at different coded levels from '0' to '-3', NEAA at '0 5' and various values of EAA. The results are shown in Table 7. The results suggests that cell density increases as EAA increases when glutamine values are low. Fig 4 shows the effect of EAA and NEAA on VCD when glutamine is controlled at coded level '-1'.

EAA values up to a 14.5 coded level (uncoded value =12.625 X) are unfeasible because of osmotic effects or inhibition of metabolic pathways due to overfeeding the nutrients. According to the above results, however, it appears that with reduced Glutamine levels, higher concentrations of EAA and NEAA at 0.5 coded level large cell densities could occur

Alternate Experiments:

To further evaluate the surface we ran some alternate experiments at different levels of Glutamine (from coded level '0' to' -3'), keeping EAA constant at coded levels 2 and 4 We expected low cell growth at a Glutamine value less than '-1 5 coded level' and no cell growth at zero ('-2' coded level) glutamine concentration. We also expected the EAA coded level of '2' to result in higher cell densities compared to EAA coded level of 4, due to osmotic effects and the inhibition of metabolic pathways from over-feeding.

Therefore 14 additional experiments were conducted, four on the ridge, four at different levels of Glutamine keeping EAA at coded level 2, four at EAA coded level 4, and two controls (Basal media) as shown in Table 8

These experiments were conducted under the same conditions as the initial experiments. The starting density of the cultures was $2*10^5$ cells/ml., and the cells were allowed to adapt to the media's in four passages The final viable cell densities were derived as an average of the third and the fourth passage, as shown in Table 8.

Media-6, had a higher cell density compared to the controls (13 and 14), but the last passage of the media's 3, 9, and 5 were nearly equal to the control media, as shown in Fig 5. These media, which had results equivalent to, or better than, Media-0, were carried out for one more passage (up to 8 days), to validate the data. The results are shown in Fig 6. From the passage 5, the viable cell density attained in the Media 6 after 5 days is about 1 6 times more than the control.

Replicate Experiments:

To validate the above results, the cells in the control media were taken out of a frozen state, and the experiment was repeated three times with control media and Media-6. Cells were allowed to go for four passages and the final viable cell densities were taken as an average of passage 3 and passage 4 The results are presented in Fig 7.

For Media-6 the viable cell density averaged over the replicates attained 1.45×10^6 cells/ml, which is 1.4 times more than the control media and within two standard errors of 1.23×10^6 cells mL from the original run of media 6 (se 2.15×10^5 cells ml for media 6). After four passages if the cells were allowed to grow to passages of 5

to 7 the viable cell density increased to 1.6×10^6 cells/ml. The Viable cell density on the control experiment in these passages (Passages 5 to 7) was between 9.2×10^5 cells/ml to 1.1×10^6 cells/ml, with in the standard deviation of 1.2×10^5 cells/ml (data not shown).

Assuming the antibody production depends on the cell density, we should see an increase in the antibody production with the increase in cell density. The production is determined and standard deviation is calculated in these media's from triplicate experiments The results are given in Fig 8. The production is determined for all the initial 50 media experiments, and the results were analyzed using SAS/STAT procedures. We saw the same trend for production also where the stationary point is a saddle point, and the ridge values for the glutamine are moving in the negative direction and in positive direction for EAA and NEAA, shown in Table 9. The R^2 value for the model was 0.75 which shows adequacy of the model in explaining the effect of the variables on the response which is antibody production.

So the antibody production in the Media 6 was 1 6 times more than the control Media. So the Composition of the variables in the best Media – 6 is Table 10.

Cost effectiveness:

There are many media's which are commercially available in the market manufactured by different biotech companies (GIBCO, IRVINE SCIENTIFIC, HYCLONE, SIGMA CAMBREX, BD BIOSCIENCES etc). These media's come in both serum free and chemically defined free of animal derived proteins. Usually media containing proteins (human, animal and recombinant) cost (60-75 $) more than protein free defined media's (55-65$). The media prepared n our facility costs 48.3 $, which is not protein free but it is serum free and chemically defined. Sixty percent of media cost is coming from ITS (which is a protein supplement). The cost of media can be reduced by decreasing the concentration of ITS in the media since from the data analysis it has been proved that ITS doesn't show any effect on the response. The cost can also be reduced by replacing the ITS (which also makes media completely protein free) with some other chemically defined components. A replacement for Insulin is still under research, while transferrin can be replaced by Sodium Iron (III) EDTA, and selenium which is a synthetic can be replaced by Sodium Selenite (34).

Conclusions:

The increase in the Viable cell density (Cells/ml), and the production of the antibody against BoNT-A was accomplished by using Box-Wilson's Central Composite Design. The viable cell density is increased by 1.4 times and the antibody production increased by 1.6 times. The lower values of the Glutamine and higher values of EAA are preferred. From the results it is expected that one of the amino acids is replacing the role of amino acid-Glutamine, and may be acting as limiting nutrient. So doing amino acid analysis, that limiting amino acid can be found, and if it is added separately, then we can decrease the concentration of the total amino acid, by that way we can make the media more economical and may also get higher cell densities and antibody production.

List of Tables

Table 1: Composition of the individual components in mg/L in the solutions EAA, NEAA, ITS, Lipids, concentrated solutions from Gibco.

(50X) EAA [mg/L]	(100X) NEAA [mg/L]	(100X) ITS [mg/L]	(100X) Lipids [mg/L]
L-arginine [6320]	L-Alanine [890]	Insulin [1000]	Arachidonic acid [2]
L-Cystine[1200]	L-Asparagine[1320]	Transferrin [0 67]	cholestrol [220]
L-Histidine*HCl*H2O [2100]	L-Aspartic acid [1330]	Selenium [0.55]	DL-alpha-tocopherol-acetate [70]
L-Isoleucine [2620]	L-Glutamic acid[1470]		Linoleic acid [10]
L-Leucine [2620]	Glycine [750]		linolenic acid [10]
L-Lysine HCl [3625]	L-Proline [1150]		myristic acid [10]
L-Methionine [755]	L-serine [1050]		oleic acid [10]
L-Phenyl alanine [1650]			palitoelic acid [10]
L-Threonine [2380]			palmitic acid [10]
Ltryptophan [510]			pluronic [0.1%]
L-Tyrosine [1800]			stearic acid [10]
L-Valine [2340]			Tween 80 [2200]

Table 1(a): Concentrations of the components in the control media.

Components	Composition (mg/L)	Components	Composition (mg/L)
CaCl2 (anhyd)	99 1	Biotin	0.02
CuSO4*5H2O	0 00125	D-Ca Pantothenate	4
FeSO4*7H2O	0 415	Choline Chloride	18
KCl	276.8	Folic Acid	4
MgCl2 (anhyd)	28 61	i-Inositol	25 2
NaCl	5500	Niacinamide	4
NaHCO3	2100	Pyridoxine HCl	4
Na2HPO4 (anhyd)	71	Riboflavin	0 4
Na2HPO4*H2O	62 5	Thiamine HCl	4
ZnSO4*7H2O	0 43	Vitamin B12	1.413
KNO3	0 038	Insulin	10
MgSO4 (anhyd)	50 8	Transferrin	5 5
Na2SeO3	0.0085	Sodium selenite	0 0134
D-glucose	**4000**	Arachidonic acid	0.014
Linoleic Acid	0.04	cholestrol	1 54
Lipoic Acid	0 105	DL-alpha-tocopherol-acetate	0 49
Phenol Red	8 1	Linoleic acid	0.07
Putrescine 2HCl	0 0805	linolenic acid	0 07
Sodium Pyruvate	110	myristic acid	0.07
HEPES	2979	oleic acid	0 07
L-Alanine	15 57	palitoelic acid	0 07
L- Arginine*HCl	221.2	palmitic acid	0 07
L-Asparagine*H2O	23 1	stearic acid	0 07
L-Aspartic Acid	23 27	Tween 80	15 4
L-Cystine*2HCl	42	pluronic	0 10%
L-Glutamic Acid	25 72		
L-Glutamine	**584**		
Glycine	13.12		
L-Histidine*HCl*H2O	73.5		
L-Isoleucine	91.7		
L-Leucine	91 7		
L-Lysine*HCl	126 88		
L-Methionine	26 4		
L-Phenylalanine	57 7		
L-Proline	20.12		
L-Serine	18.37		
L-Threonine	83 3		
L-Tryptophan	17.8		
L-Tyrosine*2Na*2H2O	63		
L-Valine	81 9		

Table 1(a):

Table 2: Actual factor levels corresponding to coded factor levels

		Actual factor level at coded factor level of:				
Level	**Symbol**	**-2**	**-1**	**0**	**1**	**2**
Glutamine	X1	1mM	2 5mM	4mM	5 5mM	7mM
EAA	X2	0 25X	1X	1.75X	2.5X	3.25X
NEAA	X3	0.25X	1X	1.75X	2 5X	3 25X
ITS	X4	0X	0 5X	1X	1 5X	2X
LIPIDS	X5	0 1X	0.4X	0 7X	1X	1 3X

Table 3: Treatment combinations with variables in coded values and the values of response.

Run	X1	X2	X3	X4	X5	Y
1	1	1	-1	-1	1	8.10E+05
2	1	1	-1	1	-1	7 20E+05
3	-1	-1	-1	-1	-1	6 50E+05
4	0	0	0	2	0	8 30E+05
5	-1	1	1	-1	-1	1 00E+06
6	1	-1	1	-1	1	8 00E+05
7	2	0	0	0	0	7 30E+05
8	0	0	0	0	2	7.10E+05
9	1	1	-1	1	1	5.40E+05
10	**0**	**0**	**0**	**0**	**0**	**9.50E+05**
11	1	-1	-1	1	1	6.60E+05
12	**0**	**0**	**0**	**0**	**0**	**9.30E+05**
13	-1	-1	1	1	1	8 40E+05
14	1	-1	1	1	-1	8 80E+05
15	1	-1	1	1	1	6 25E+05
16	-1	-1	1	-1	-1	7 00E+05
17	**0**	**0**	**0**	**0**	**0**	**1.18E+06**
18	-1	-1	-1	1	1	8.15E+05
19	0	0	0	0	-2	1.15E+06
20	1	-1	-1	1	-1	6 55E+05
21	**0**	**0**	**0**	**0**	**0**	**1.16E+06**
22	-1	-1	-1	-1	1	2 00E+05
23	-2	0	0	0	0	1 52E+06
24	0	0	0	-2	0	5 15E+05
25	0	-2	0	0	0	3 10E+05
26	-1	-1	1	1	-1	8 20E+05
27	1	-1	-1	-1	1	5 10E+05
28	1	1	1	-1	-1	8 10E+05
29	-1	1	-1	-1	-1	7 85E+05
30	**0**	**0**	**0**	**0**	**0**	**1.18E+06**
31	-1	-1	-1	1	-1	8 30E+05
32	0	0	2	0	0	9 55E+05
33	-1	1	1	-1	1	1 29E+06
34	0	0	-2	0	0	1 80E+05
35	1	1	-1	-1	-1	2 00E+05
36	1	1	1	-1	1	2.20E+05
37	**0**	**0**	**0**	**0**	**0**	**1.21E+06**
38	-1	1	1	1	1	1 03E+06
39	-1	1	-1	1	-1	9 00E+05
40	1	-1	1	-1	-1	9 85E+05
41	-1	1	1	1	-1	8 65E+05
42	1	-1	-1	-1	-1	8 75E+05
43	1	1	1	1	-1	7.70E+05
44	1	1	1	1	1	1.08E+06
45	**0**	**0**	**0**	**0**	**0**	**9.90E+05**
46	-1	1	-1	-1	1	9 35E+05

47	-1	1	-1	1	1	8 55E+05
48	-1	-1	1	-1	1	1 00E+06
49	0	2	0	0	0	8 90E+05
50	**[redacted]**	**0**	**0**	**0**	**0**	**9.85E+05**

Where α = '2'. Response = Viable cell density in cells/ml. '-1', '0', '+1' are coded factorial levels.

Table 4: The't' and 'p' values of full model with X1, X2, X3, X4, X5 as independent variables

Parameter	t Value	p Value
Intercept	13 75	<0 001
X1	-2 79	0 0092
X2	1 53	0 1376
X3	3.07	0 0046
X4	1 2	0 2404
X5	-0.81	0.425
X1*X1	0 66	0 5167
X2*X1	-2 1	0 1446
X2*X2	-2 67	0.0124
X3*X1	-0 27	0 7855
X3*X2	-0.1	0 9206
X3*X3	-2 86	0 0078
X4*X1	0 31	0 758
X4*X2	0 22	0 8304
X4*X3	-0 74	0 4662
X4*X4	-2 21	0 0352
X5*X1	-0 8	0 4292
X5*X2	1 3	0 2026
X5*X3	0 25	0.8061
X5*X4	0.2	0.8427
X5*X5	-0 58	0.5675
Linear		0 0046
Quadratic		0 0055
Cross Product		0 6621

R^2 0.6339 for the total model

Table 5: The 't' and 'p' values of the reduced model with X1, X2, X3 as independent variables.

Parameter	t value	p value
Intercept	15 56	<0 0001
X1	-2 81	0 0076
X2	1 54	0 132
X3	3 1	0 0036
X1*X1	0 66	0 5123
X2*X1	-2.11	0 0407
X2*X2	-2 69	0 0105
X3*X1	-0 28	0 7835
X3*X2	-0 1	0 9199
X3*X3	-2 88	0 0064
Linear		*0 001*
Quadratic		*0 0035*
Cross Product		*0 2242*

R^2 - 0.5022 for the reduced model

Table 6: Ridge of steepest ascent for X1, X2, X3 independent variables, and estimated response and standard error

X1	X2	X3	Estimated Cell Density (10^5)	Std.Err
-0 15	0 07	0 11	10 1	62346
-0 33	0 13	0 18	10 4	61381
-0 52	0 2	0 23	10 7	60421
-0 71	0 26	0 26	11	60558
-0 91	0 32	0 28	11 4	63278
-1 1	0 38	0 3	11 8	70025
-1.29	0 43	0.31	12 2	81626
-1.49	0 49	0.33	12 7	98153
-1.68	0 55	0 34	13 2	119266
-1.87	0 6	0 35	13 7	144554

Table 7: Effect on EAA, NEAA and VCD when Glutamine is controlled at different coded levels

X1(level)	X2(Level)	X3(Level)	VCD
-0 5	4.5	0 5	1 27E+06
-1	6.5	0 5	1 56E+06
-1 5	8 5	0 5	1 95E+06
-2	10 5	0 5	2 43E+06
-2 5	12 5	0 5	3 01E+06
-3	14 5	0 5	3 69E+06

Table 8: Alternate experiments carried out on ridge and glutamine controlled at different coded levels, keeping EAA constant at coded level 2 and coded level 4.

Media	X1	X2	X3	VCD (105)	Standard Deviation
1	-0 52	0.196	0 225	8 13	203490
2	-1 1	0.375	0.299	8 81	179368
3	-1 487	0 491	0 329	8 61	60052
4	-1 874	0 604	0.352	8 01	152664
5	0	2	0 5	9 35	42230
6	-1	2	0 5	1 23	**215928**
7	-2	2	0 5	7.23	277564
8	-3	2	0 5	1.79	45162
9	0	4	0 5	7.93	102429
10	-1	4	0 5	4 49	152555
11	-2	4	0 5	5.7	144684
12	-3	4	0 5	2	52915
13	0	0	0	9 15	134722
14	0	0	0	9 48	121484

Table 9: Ridge of steepest ascent of the reduced model for getting maximum antibody production with independent variable X1, X2, X3, X4 and X5 kept constant at their zero-level

X1	X2	X3	Estimated Response (mg/L)
0	0	0	12 73
-0 172	-0 01	0 101	13.46
-0 34	-0 001	0 2	14 21
-0.522	0 03	0 293	15
-0 698	0 08	0 38	15 82
-0 874	0.149	0.461	16 69
-1 049	0 226	0.536	17 61
-1 22	0 31	0 607	18 58
-1 39	0 4	0 673	19 6
-1 56	0 496	0 736	20 6
-1.73	0 595	0 796	21 8

Table 10: Concentrations of the five variables of the optimal media, (total concentration of all amino acids in EAA and NEAA are given in g/L). Concentration of the remaining components same as given in Table 1(a).

Variables	Concentration
Glutamine	2 5 mM
EAA	3 25 X (1 67 g/L)
NEAA	2 125 X (0 168 g/L)
ITS	1 X (0.22 g/L)
Lipids	0.7 X (0.71 g/L)

List of figures:

Figure 1:

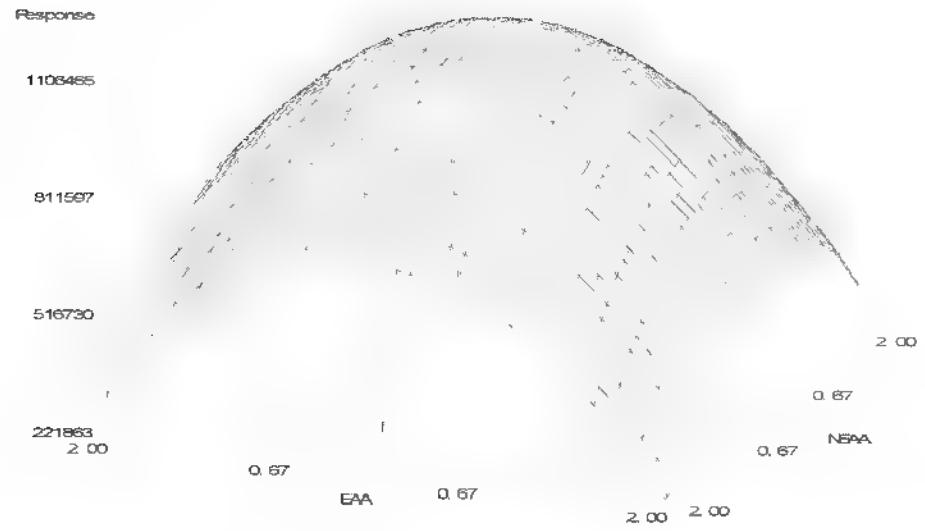

Fig 1: Response surface plot showing the effect of EAA, NEAA, and their mutual effect on the Response (Viable cell density). Other variables are held at zero level.

Figure2:

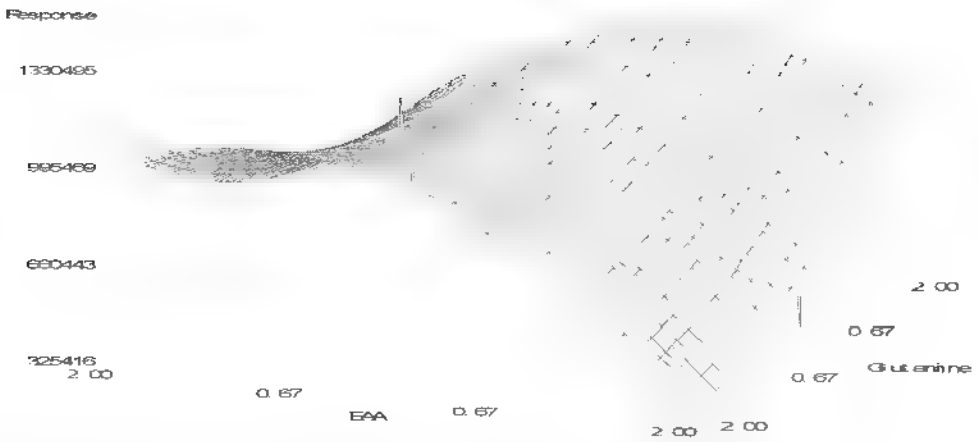

Fig 2. Response surface plot showing the effect of EAA, Glutamine and their mutual effect on the Response (Viable cell density). Other variables are held at zero level.

Figure 3:

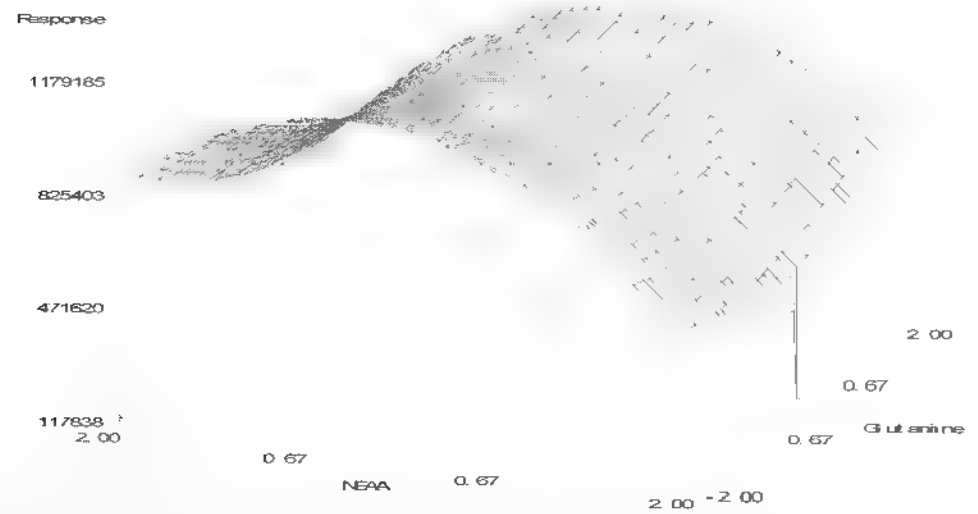

Fig 3: Response surface plot showing the effect of NEAA, Glutamine and their mutual effect on the Response (Viable cell density). Other variables are held at zero level.

Figure 4:

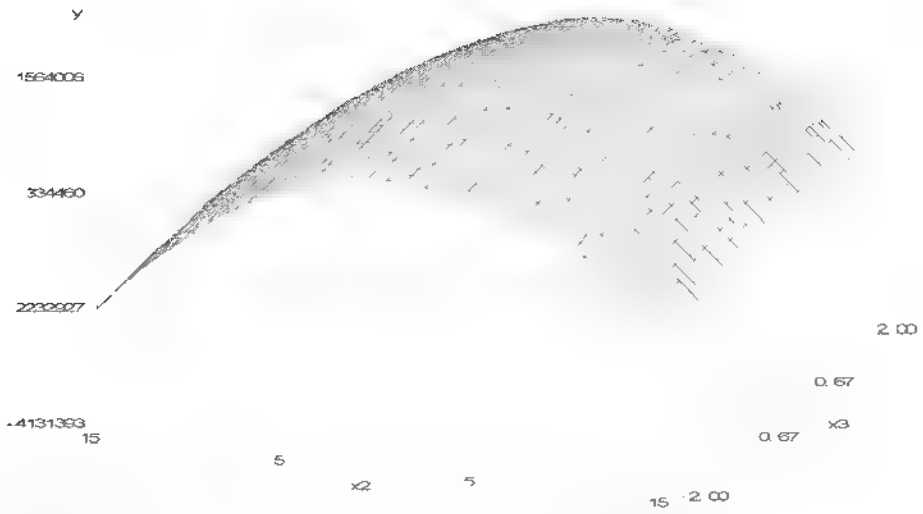

Fig 4: Response surface plot showing the effect of EAA (X2), NEAA (X3) and their mutual effect on the Y (Viable cell density), when Glutamine (X1) is controlled at '-1' coded level.

Figure 5:

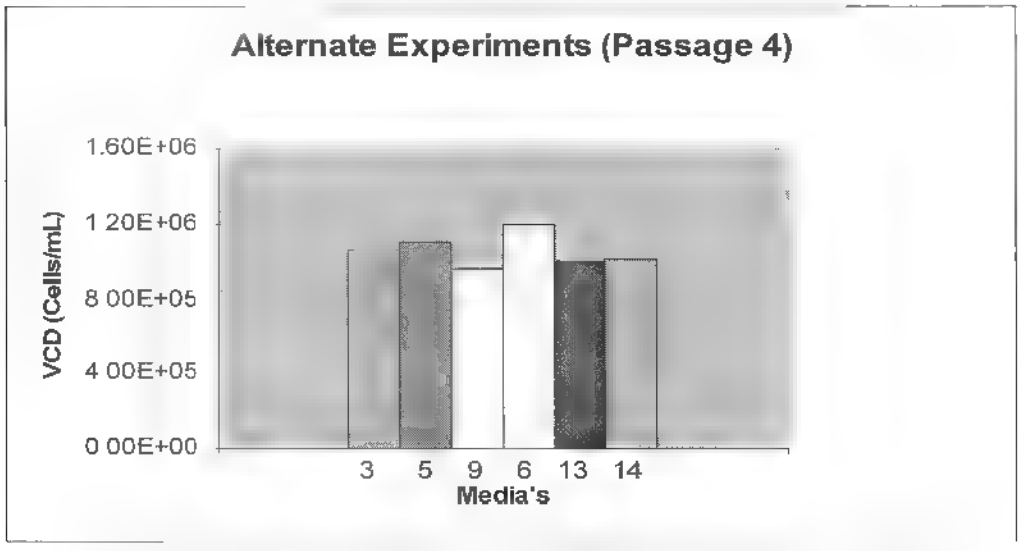

Fig 5: Results of the Paasage #4 alternate experiments carried to further explore the surface

Figure 6:

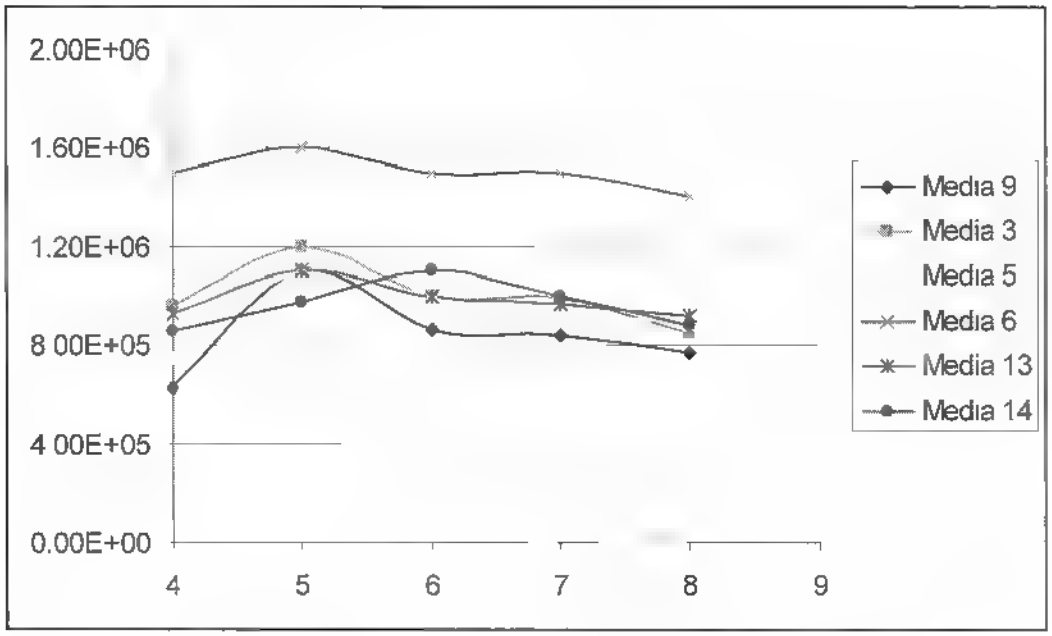

Fig 6: Results of the Paasage #5 alternate experiments carried to further explore the surface

Figure 7:

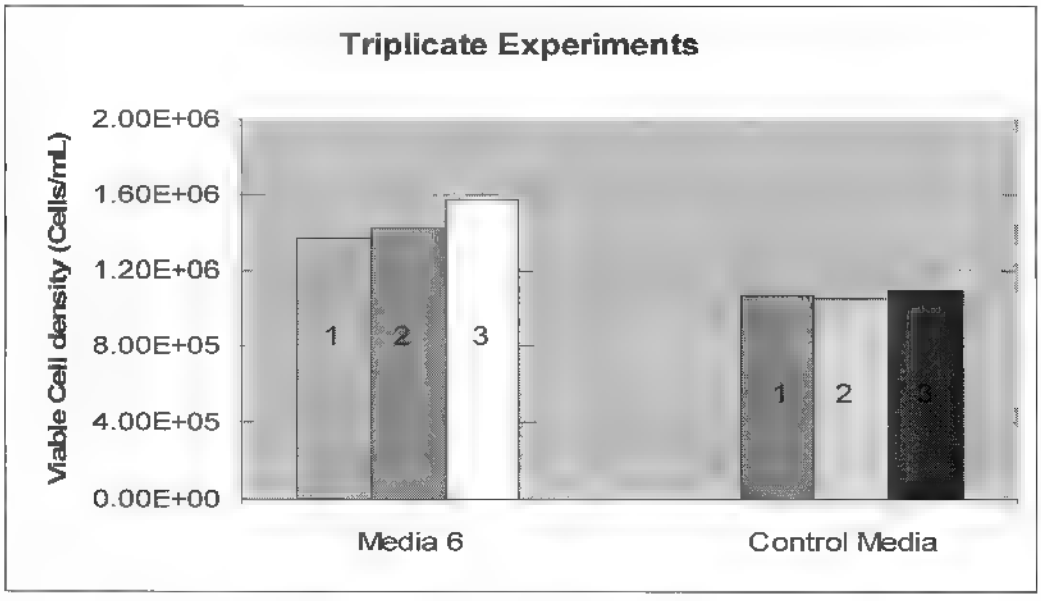

Fig 7: Results of the triplicate experiments of Media 6 and the Control Media.

Figure 8:

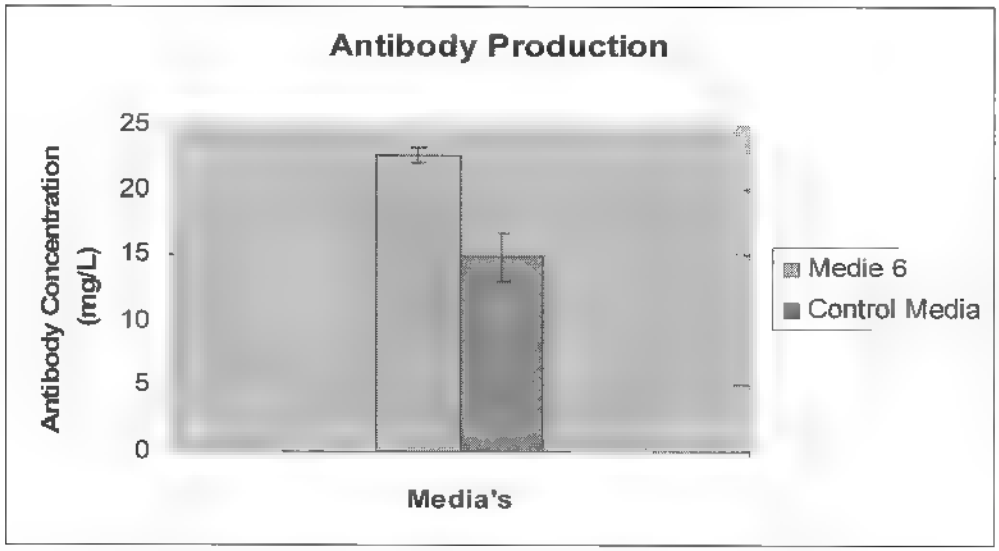

Fig 8: Antibody production in Media-6, and the Control Media.

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Fed-Batch process development for Cultivation of CHO Cells producing antibody against Botulinum Neurotoxin Serotype A

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Abstract

Substrate controlled fed-batch cultures were conducted to study the effect of growth and antibody production in Chinese hamster ovary cells. The combined effect of glucose and glutamine-controlled fed-batch cultures controlling glucose at 2 g/L and glutamine at zero, 0.5, and 2 mM/L, with varying concentrations of the glutamine in the feed, were compared The inhibitory metabolites ammonium and lactate formation were reduced by controlling glucose and glutamine at low values. The experiments were performed at bench scale in 1 L bioreactors. The experiments were carried out in three different sets, with each set having three experiments where glutamine was controlled at zero, 0.5, and 2 mmol/L. The basal medium used in the fed-batch studies was optimized initially in batch conditions, and the feed mediums used were the same as the basal medium (excluding glucose and NaCl) concentrated in essential amino acids and glutamine. The glutamine concentrations used in feed mediums were 25 mM, 7.5 mM and 15 mM (10x, 3x, and 6x concentration of glutamine, respectively, in the basal medium) in three different sets and glucose was controlled through a separate feed, with the glucose solution concentrated at 30 g/L. In the three sets of experiments, the concentration of essential amino acids was 10x of the 50x solution from Gibco. The culture volume appears to have a significant effect in achieving the optimal growth conditions. The fed-batch cultures controlled at 0.5 mM were more comparable than the fed-batch cultures controlled at zero and 2 mM in all three sets, due to the addition of an optimal feed volume to the bioreactor. The optimal feed volume prevented the formation of growth-requiring nutrients and inhibitory metabolites in the culture The optimal fed batch process yielded a maximum viable cell density of 2.54×10^6 cells/mL, a 44 6% improvement over the batch process while the final antibody concentration attained was 61.07 mg/L, a 31% improvement.

Introduction

The performance of successful fed-batch cultures depends on feeding a nutrient concentrate to maintain cell viability and antibody production. Glucose and glutamine are the primary carbon, nitrogen and energy sources in mammalian cells (Zielke, 1978). At higher glucose and glutamine concentrations, however, cells tend to produce higher levels of the inhibitory metabolites ammonia and lactate, which in turn inhibits cell growth and protein production (Glacken, 1986). The quality of the protein is also affected by the higher levels of inhibitory metabolite accumulation (Anderson, 1995). If concentrations of glucose and glutamine are controlled at low levels, then there will be less accumulation of waste products as well as more efficient use of the medium components, resulting in higher viable cell densities and protein production (Glacken, 1986). Variations in limiting nutrient levels have been correlated with variable specific productivity and heterogeneous post-translational modifications (Van Erp, 1991).

Fed-batch cultures are usually grown as batch cultures before feeding of the nutrient concentrate has been initiated. Addition of nutrients like glucose and glutamine before cells reach the stationary phase has resulted in a 1 9- to 5-fold increase in monoclonal antibody production relative to batch cultures (Reuveny, 1986; Flickinger, 1990) Addition of more complex nutrient feed solutions has resulted in an 11-fold increase of antibody titers compared to batch cultures (Robinson, 1994). In animal cell cultivation, the consumption rates of glucose and glutamine are usua ly higher than the consumption rates of the other nutrients. Because these two nutrients usually

limit cell growth and protein production (Lindell, 1992,. simple fed-batch strategies, e.g. supplementing the culture with glucose or glutamine as the cells reach the stationary phase, have been used However, current analytical techniques allow for analysis of the spent culture medium to determine nutrient utilization and identify limiting nutrients. Analysis of the spent culture medium for glucose, typically the main carbon source, can be performed using automated analyzers, while amino acid concentrations can be determined by HPLC (Reid et al., 1987). Analysis of other nutrients such as vitamins, lipids, proteins, and other trace elements is more challenging and time-consuming. Because of these new analytical techniques, initial simple feeding strategies have evolved into more complex strategies employing multi-component feeds at various stages of cell growth. Several investigators (Flickinger, 1990; Robinson, 1994a; Bibila, 1994a) have reported on the beneficial effects of feeding glucose, glutamine, and concentrated amino acid solutions to MAB-producing cultures, achieving final titers up to 600 mg/L, a 2- to 4-fold improvement over the batch culture Although the strategies employed vary, each relies upon a combination of physiological reasoning, nutrient depletion analysis and iterative feed design to maximize cell growth, culture longevity, and MAB production.

In this article, we describe a nutrient feeding strategy to achieve a highly viable cell concentration and MAB production. This is our initial attempt to find the optimal feed and basal concentrations of the nutrients. Based on offline measurements of glucose and glutamine and estimation of the nutrient consumption rate, a stoichiometric amount of nutrients was fed to sustain cell growth and minimize the metabolite accumulation. In this method, we used the same feed medium as that of the basal medium concentrated in glutamine, glucose and amino acids. Glucose was kept constant and glutamine was controlled at various levels in the basal medium At the same time the concentration of the glutamine in the feed medium was varied in order to find the optimal basal and feed glutamine concentrations. This method of manipulating cell metabolism through offline control of nutrient feeding rates was found to be effective

Materials and Methods

Cell line Development

The cell line used in this study was the CHO-DG44 [dhfr (-)] producing pS25-IgG monoclonal antibody that neutralizes Botulinum Neurotoxin-A To obtain highly expressed cell lines, cells were transfected with a plasmid containing the light and heavy chains of the S25 monoclonal antibody, in addition to the gene dihydrofolate reductase (Dhfr) Dhfr has been used in Chinese hamster ovary cells to increase production of recombinant proteins and monoclonal antibodies (Alt et al., 992). CHO-DG44 cells, which lack the gene for Dhfr, were obtained from Larry Chasin (Columbia University). This cell line allows for gene amplification through Dhfr expression, which increases the number of copies of plasmid DNA.

The CHO-DG44 cell line was transfected with the pS25 plasmid (Fig. 1) using Lipofectamine 2000 (Invitrogen), which is an efficient chemical method of transfection. Cells were seeded with 0.5 ml in 24 cell plates at 2 x 10^5 cell/ml in α-MEM media with 8% FBS and grown overnight. 1 µg plasmid DNA and 0.5-5.0 µl Lipofectamine 2000 were combined in 0.1 ml Opti-MEM media (Invitrogen) and allowed to equilibrate for 20 min

0.5 to 2 ml of Lipofectamine 2000 proved to be optimal. Plasmid DNA was added to the transfection mix, which was either uncut or linearized with NruI. This site was chosen since it would place the amplifiable gene (Dhfr) between the heavy and the light chains. The DNA/Lipofectamine 2000 solution was added to the 24 well plates and the plates were incubated at 37°C overnight. Stably transfected cells were selected in α-MEM media lacking ribonucleotides and deoxyribonucleotides (α-MEM(-)) and with 8% FBS, which prevents cells lacking Dhfr from growing. Cells were passaged several times and individual clones were obtained by dilution cloning at 0 5 cells/well in 96 well plates. Samples were taken from wells containing growing cells after 16 to18 days. The antibody concentrations were determined using an ELISA specific method for the Fc portion of a human heavy chain of IgG Sixteen cell lines showed antibody titers greater than 0.1 mg ml, while one cell line showed a concentration greater than 2 μg ml. This clone (CHO-DG44 S25 #56) had the highest expression level throughout the selection process. This clone contains the plasmid that is uncut and not linearized Presence of the light chain was later confirmed by both Western blotting and a human kappa chain specific ELISA method. The cell line CHO DG44 S25 #56 had light and heavy chain concentrations greater than 2 μg ml and was therefore used for the production of S25 antibody in suspension culture. Cells (CHO-DG44 S25 #56) were scaled up from 96 well plates to 25 cm2 T-flask, 75 cm2 T-flask and finally into suspension culture. Cells were weaned off from serum (α-MEM(-)) and with 8% FBS) to serum-free in CHO-S-SFM II media, and then adapted from CHO-S-SFM II into our chemically-defined basal media.

Basal Medium

The first step in the fed-batch process design is to develop or identify a near optimal basal medium (Robinson et al., 1994a). Nutrients are then maintained at constant concentration during the course of the culture by adding concentrated nutrient solutions designed to match nutritional requirements of the cells as determined by analysis of the spent culture medium.

A near-optimal medium CHO-OPTIMAL was derived from CHO-ZERO (original medium), in a batch culture, which started from a serum-free IMDM: HAM'S F12 (1:1) excluding Hypoxanthine and Thymidine. The near-optimal medium was developed at home in order to find the optimal concentrations of glutamine, Essential Amino Acids, Non-Essential Amino Acids, ITS (Insulin, Transferrin, Selenium), and Lipid supplements, which served as the variables for this study. Results were analyzed using Response Surface Methodology (RSM). The optimal concentrations of the variables studied were 2.5 mM Glutamine, 7.4 mM (2X) EAA, 1.4 mM (0 5X) NEAA, 1X ITS supplement, and 0.7X Lipids supplement CHO-OPTIMAL was used as basal medium in the fed-batch culture studies.

Feed Medium

A successful fed-batch culture depends strongly on the nutrient environment. An investigation of the optimal nutrient environments is complicated because of the interrelated effects of various factors Creating an optimal nutrient environment depends on two issues. 1) the optimal nutrient composition for the cell culture (i.e., the optimal basal media), and 2) the rate of consumption of the individual nutrients by cells. The consumed nutrients

should be continuously supplemented into the bioreactor during the fed-batch culture. The amount of one nutrient to be added depends on its consumption rate which, in turn is a function of the cell density, growth and metabolism.

We addressed the first issue by developing a near-optimal media in batch optimization studies and the media is used as a basal media for the fed-batch studies. In mammalian cell cultures, glucose, glutamine and amino acids are consumed in large amounts, resulting in proteins, lactate, ammonia, alanine, and others Therefore, the feed medium we used was the same as the basal media concentrated in glucose, glutamine, and essential amino acids. Three different feed media (FM1, FM2 and FM3) were prepared, each with varying concentrations of glutamine. The concentration glutamine in FM1 was 10× the concentration in the basal media. In FM2 the concentration was 3× the concentration of the basal media, and in FM3 it was 6× the concentration of the basal media. FM1, FM2, and FM3 did not contain sodium chloride Essential amino acid concentrations were diluted with a 50X concentrated solution from Gibco. Glucose feed was completely separated from these feed media, the concentration of which was a 30 g/L solution prepared in water. The feed was added to the reactors through a completely differently feed line.

Bioreactor

Experiments were carried out in 1-L Das-Gip bioreactors having a working volume of 600 ml. We assume the working volume to be 400 mL to ensure uniform agitation The entire system comprises a culture system, gassing system, monitoring system, and dosing system. The culture system consists of a temperature-controlled incubator with 8 vessels that is placed on magnetic stirrer platform and equipped with pH and pO_2 electrodes and feed and air supply/removal connections. The culture vessels have glass impellers and the stirring rate is maintained constant at 60 rpm. The gassing system of the Das-gip provides an individual mix of up to four gases to each culture vessel (air, O_2, CO_2, N_2) CO_2 and O_2 gas concentrations were controlled by feedback from pH and pO_2 electrodes. Each vessel has an electronic mass flow control mechanism and a gas totalizer function. The monitoring system processes the signals from the pH and pO_2 electrodes At the same time, it regulates pH and pO_2 in the medium by adjusting the CO_2 and O_2 in the gas mixture to maintain the set points, which were 7.0 with 50 % saturation in our experiments. The dosing system offers an individual and regulated delivery of feed medium. The dosing proceeds according to the user- defined profiles or is fully automated based on the online-determined OUR (Metabolic Activity based-feeding). The control system configures the pH and pO_2 gassing and dosing with user-defined profiles, and calibrates the electrodes and dosing system. The analyses of the logged data are performed using the Gas-Dip Chart Wizard for MS Excel on a Microsoft Windows platform.

Control strategy of fed-batch culture

Basal medium was filtered into the bioreactors using 0.2 μm-rated membrane bottle top filters The actual working volume for the reactors is 600 ml, but we assumed the reactor working volume to be 400 ml in order to ensure perfect mixing of the nutrients with glass ball impellers at 60 rpm. The feed medium was filtered into 250 ml sterile glass bottles, stored at 4^0C and aseptically connected to respective bioreactors. The seeding density for the bioreactors was 2×10^5 cells/ml and initial volume was 220 mL Sampling of the reactors was performed every 20 to 24 hours, and the results were analyzed. The viable cell density was measured by a hemacytometer using the tryphan-blue exclusion method. A NOVA analyzer was used to determine the concentration of the nutrients, glucose,

glutamine, ammonia, lactate, and osmolarity of the medium. The addition of the feed medium for the reactors was started after approximately 36 hours of inoculation The feeding protocol was based on maintaining the desired post-glucose and glutamine concentrations. As soon as the reactors were sampled, concentrations of the glucose and glutamine were found using a NOVA analyzer. In addition, we calculated the volume of the feed medium that needed to be added to reach the desired set points for glucose and glutamine using the following equations: Volume

of feed (gluc/gln) (ml) [V_{feed}] $$\left[\frac{(gluc/gln)_{set} - (gluc/gln)_{net} * V_{Reactor}}{(gluc/gln)_{feed}} \right] \quad (1)$$

where $(gluc/gln)_{set}$ – desired set point of glucose/glutamine

$(gluc/gln)_{net}$ – current value of glucose/glutamine from NOVA

$V_{Reactor}$ = Volume of reactor after sampling

$(gluc/gln)_{feed}$ – glucose/glutamine concentration in feed

Correcting for volume change in the reactor by the addition of feed, we get:

Final volume of feed to be added [V_{final}] = $$V_{feed}\left[1 + \frac{V_{feed}}{V_{Reactor}}\right] \quad (2)$$

The pumps of the dosing system were turned on manually at the maximum flow rate of 40 ml/hr, and the required volume of the feed was added to reach the set desired set points within one hour after sampling. The flow rates of the pumps for the next 24 hours (until the next sampling) were calculated based on the previous growth rate and the specific consumption rates of glucose and glutamine from the batch data. The specific consumption rate for glucose and glutamine between the two time intervals t_1 and t_2 were calculated using the following equation.

Sp.glutamine consumption rate (q_{gln})

$$\left[\frac{(((gln)_1 V_1 - (gln)_2 V_2) + V_F (gln)_{feed}(t_2 - t_1))/(V_2(t_2 - t_1))}{((X_1 + X_2)/2)}\right] \quad (3)$$

The consumption rate for glucose was calculated in the same way, noted as (q_{gluc}),

where

$(gln)_1$, $(gln)_2$, $(gln)_{feed}$ = concentration of glutamine at time intervals t_1, t_2, and concentration of feed respectively.

V_1, V_2 = Volume of the reactors at two time intervals t_1, t_2, respectively

X_1, X_2 Viable cell densities at intervals t_1, t_2

V_F = Total volume of feed added between intervals t_1, t_2

The consumption rate of the glucose was assumed to be constant throughout the feeding. The glucose feed solution, which was separated from the second feed solution (basal media concentrated in glutamine and amino

acids), was based on a consumption rate calculated using data from previous batch studies The glucose calculated consumption rate was $9.58*10^{9}$ mg cell/hr

The consumption rate of the glutamine was assumed to vary over time according to desired set points. The set points of glutamine used in our experiments were 0 2, 0.5, and 2 mM. From the previous data of batch and fed-batch experiments, we derived an exponential equation for glutamine consumption rates using Equation (3) and calculated (q_{gln}) at different time intervals.

The exponential equations used for set points 0.2, 0.5 and 2 were:.

$(q_{gln})_{0.2} = (7\times10^{9})\times t^{-1.27}$ ----------→ (a)

$(q_{gln})_{0.5} = (4.7\times10^{-9})\times t^{-1.15}$ ----------→(b)

$(q_{gln})_{2} = (2.5\times10^{-9})\times t^{-0.9}$ ----------→ (c)

The specific growth rate was calculated using the following equation:

$$\mu = \frac{dX}{dt}\cdot\frac{1}{X}+\frac{F}{V} \quad (hr^{-1}) \qquad (4)$$

where X = Viable cell density at time't' (cells/ml)

F – feed flow rate (ml/hr)

V Volume of the reactor (ml)

We assumed the endogenous metabolism or death rate to be negligible when compared to growth rate ($k_d <<< \mu$).

We assumed the growth rate calculated between previous time intervals, was assumed to be constant for the next time interval (24 hours), and calculated the predicted viable cell density using Equation (4).

Integral viable cell density was calculated between time 't_1 t' and t_2 – t+24, using Equation (5):

$$\int_{t_1}^{t_2} XV\,dt = (XV)_{ave}(t_2 - t_1) \qquad (5)$$

$$(XV)_{ave} = \frac{(XV)_1 + (XV)_2}{2} \qquad (6)$$

Using Equation (5) and Equations {a,b,c}, the required glucose glutamine concentration was calculated for the next 24 hours. The feed flow rates were calculated taking into account the effect of volume change caused by the addition of glucose and glutamine feed instantaneously, as well as the change in volume caused by the feed for the next 24 hours. The pumps were turned on manually for each reactor and left at calculated flow rates for 24 hours.

Determination of Antibody Concentration by the ELISA method

Antibody concentration was determined using the ELISA method. Affinity purified rabbit anti-human IgG antibody was diluted to 5 µg/ml in coating buffer (100 mM $NaHCO_3$ and 100 mM NaCl (pH 9.3)), and 100 µl was added to the wells of 96 well plates and incubated overnight at 4°C. The plates were washed twice in a Tris buffer (20 mM Tris-HCl, 50 mM NaCl, pH – 7.2) containing 0 1% Tween 20, and then twice in the Tris buffer alone. Blocking buffer (a Tris buffer with 0.5% BSA) was added to the 96 well plates and incubated at 37°C for 1 h.

Supernatant samples were diluted in the blocking buffer and samples were loaded into the 96 well plates in triplicate. Plates were incubated for 1 h at 37°C and the same washing procedure was repeated. 100 µl of a goat anti-human IgG-HRP conjugate antibody diluted to 0.5 to 2 mg/ml in the dilution buffer was added to the plates. The plates were incubated for 1 h at 37°C and the washing procedure was repeated. Finally, 100 µl of 50 µg/ml ABTS in ABTS buffer (Roche) was added to the plates. The absorbance was determined to be 405 nm using an ELx800 plate reader (Bio-Tek).

Results and Discussion:

The basal media used for the fed-batch studies was optimized initially in batch conditions. The culture lasted for 192 hours; the maximum cell density was attained at 120 hours, and viability started dropping significantly after then. The decreased viability occurred because glutamine, the growth-requiring nutrient and major source of energy, was depleted We also expected that some of the amino acids would behave as limiting nutrients. The maximum concentrations of viable cell density and antibody attained in batch culture were 1.45×10^6 cells.mL (on day 5) and 24 25 mg/L, respectively. The batch culture profiles are shown in Fig. 2.

The fed-batch culture was started with CHO-OPTIMAL, having initial glucose glutamine concentrations of 2.5mM and 4 g/L, respectively. It was reported that glutamine-controlled fed-batch cultures results in higher yields, reducing the inhibitory ammonium ion concentrations and overflow metabolism of the amino acids in mammalian cell cultures (Ljunggren and Haggstron, 1992). Hayter (1992) and Omasa (1992) controlled inhibitory lactate formation in glucose controlled fed-batch culture. Kurokawa (1994) reduced the production of both ammonia and lactate and increased the cell density and antibody production by controlling glucose and glutamine at low concentrations in dual substrate controlled fed-batch culture. We decided to carry out the fed-batch culture keeping glucose at low value of 2g.L and glutamine at zero, 0.5, and 2 mmol/L by supplementing the basal medium with feed mediums concentrated in glucose, glutamine and amino acids The experiments were carried out in three different sets, with three different feed mediums (FM1, FM2, FM3) varying in concentrations of glutamine

First set of experiments:

In the first set of experiments, the basal medium was CHO-OPTIMAL. The concentration of the nutrients is given in Table 1. The feed medium was FM1 with a glutamine concentration of 25mM, 10 times more concentrated than the basal medium, and a 10X concentrated essential amino acids solution diluted from a 50X concentrated solution from Gibco, [Invitrogen??]. The concentration of the individual nutrients in the feed mediums is given in Table 2. The feeding strategy was based on the offline measurements of glutamine and glucose, and from the estimation of glutamine consumption rates using Equations a, b, c, (derived from Equation (3) for different glutamine set points) and the consumption rate of the glucose from Equation (3).

The control of the culture at zero mmol/L was extremely difficult After glutamine dropped to zero, feeding of the feed medium was done equal to the calculated glutamine consumption rate, assuming that the added glutamine would be consumed by the growing cell density and that glutamine would be zero until the next sampling interval.

The estimation was not perfect, however, resulting in glutamine values only slightly higher than zero mmol/L. The control of glucose was done at 2 g/L by adding feed medium concentrated to 30 g/L. The control profiles for glucose and glutamine are shown in Fig.3. The volume of the feed medium added to the culture controlled at zero mmol/L, (total 11.6 mL) was substantially less. It was expected that the culture would be deprived of essential amino acids and other nutrients. The viable cell density attained was less when compared to the culture control ed at 0.5 mmol/L, where 57.76 mL of feed medium was added. The viable cell density profiles are shown in Fig. 4. The total antibody concentration in the culture controlled at 0.5 mmol/L was 8 11 mg, only slightly higher than zero mmol/L (6.29 mg). This is because the viable cell density dropped without maintaining the stationary phase, whereas the culture at zero mmol L had little extended stationary phase, as shown in Fig. 4 In the case of the culture controlled at 2 mmol/L, the volume of feed added (102 27mL) was more, compared to zero and 0 5 mmol/L The viable cell density attained was significantly less because the culture was overfed. In addition, the formation of ammonium (more than in the zero and 0 5 mmol/L controlled cultures) inhibited the cell growth. The ammonium would have formed from the cell metabolism and from the degradation of unused glutamine in the culture. The total antibody concentration (5 6 mg) was also very low because of lower cell densities attained in the culture. The ammonium profiles for the first set of experiments are shown in Fig. 7.

Second set of experiments:

The second set of experiments was designed based on the results achieved in the first set Since the volume of the feed medium (FM2) added to the cultures controlled at zero and 0 5 mmol/L in the first set of experiments was much smaller, we reduced the concentration of the glutamine to 7.5 mM (3 times more concentrated to the glutamine in the feed) in the feed medium, keeping all the other nutrients at the same concentration as the feed medium FM1. The concentrations of the feed mediums are given in Table 2. The feeding of the feed medium was based on the consumption rates of glutamine calculated from Equations a, b, c derived from Equation (3). Since we reduced the concentration of the glutamine in feed, we planned to add more feed volume but not limit the culture to any essential amino acids and other nutrients. The total volume of the feed added for the cultures controlled at zero, 0 5, and 2 mmol/L was 24, 216.27, and 267.61 mL, respectively The viable cell density profiles improved significantly compared to first set of experiments, as shown in Fig. 5. The viable cell density in 2 mmol/L was slightly more compared to zero and 0.5 mmol/L. The over-dilution of the culture also diluted the inhibitory metabolites (shown in Fig. 7), resulting in a favorable environment for cell growth. In the case of 2 mmol/L, the volume exceeded the maximum reactor volume, so we had to shut off the feed flow (both glucose and glutamine feeds) after 138 hours. Adding more volume of feed in the case of 2 mmol/L and 0.5 mmol/L resulted in providing more amino acids and other nutrients to the cells, which in turn resulted in extended stationary phases, increasing the ant body productions of the cultures. The total antibody concentration attained in 2 mmol/L (14 2 mg) and 0 5 mmol/L (13.02 mg) were higher than zero mmol/L (6.8 mg), as shown in Fig. 5, as well as the concentrations attained in the first set of experiments. This clearly shows the effect of the feed volume added to the culture. The more feed volume added, the higher the concentrations of amino acids and other nutrients in the medium. These higher concentrations increase the

stationary phase, the osmolarity of the medium, and also provide building blocks for protein, resulting in higher antibody concentrations.

Third set of experiments:

Considering the importance of the feed volumes to be added and the control of the inhibitory ammonium formation, we designed the third set of experiments to meet the following requirements. From the first set, we found that glutamine at 25 mM (10 X the basal glutamine concentration) was very high, resulting in limitation of the cultures for amino acids and other nutrients by adding very low volumes of feed. In the second set of experiments, we found that glutamine at 7.5 mM (3X the basal glutamine concentration) resulted in over- dilution of cultures, which helped to increase the stationary phase, but not to attain the higher viable cell densities. Therefore, we expected that the optimal glutamine concentration in feed was between 25 mM and 7.5 Mm, and we decided to use the glutamine concentration of 15 mM (6X the basal glutamine concentration). By using this concentration of the glutamine in the feed, we expected to meet the requirements of providing enough nutrients to the cells by adding optimal volumes of the feed to the cultures.

Better results were achieved from the third set of experiments than in previous sets, both in the total viable cell densities and in total antibody concentration. The maximum antibody concentration was attained in the culture controlled at 0.5 mmol/L (15 6 mg), achieved by increasing the period of both the exponential and stationary phases The volume of the feed added (97.43 mL) was in between the volumes of the feed added for cultures controlled at 0.5 mmol L in previous sets. We expected that the culture would not be deprived of essential nutrients required for cell growth and antibody production The performance of the culture at 0.5 mmol/L was more optimal than zero and 2 mmol/L in cell densities and antibody formation, as can be seen in Fig. 6. This is because cells prefer to grow well in cultures controlled at low glutamine values, which decreases the formation of ammonia, the main inhibitory product (see Fig. 7) In the case of the culture controlled at 0.5 mmol/L, the feed volume added was optimal, not resulting in over-feeding (as in the case of 2 mmol/L, where the volume added was 197 33 mL) and limitation of any nutrients (as in the case of zero mmol/L, where 25 mL was added) in the culture, providing an optimal environment for cell growth and antibody production. The maximum viable cell density attained in the fed-batch culture controlled at 0.5 mmol L was 44.6 % more, and maximum antibody production was 31% more, than concentrations attained in the batch cultures

Comparison to different fed batch process yields in literature:

The final antibody concentration achieved in the fed batch was 61.07 mg/L which was 2.8 folds higher than batch culture But today highly sophisticated fed-batch strategies have evolved resulting in higher antibody titers of 1-2 g/L (Table 3). Early efforts to extend culture longevity focused on supplementing the basal medium with single limiting nutrient, but now a day's capability of the detailed analysis of the spent medium has led to the highly fortified basal medium enriched in multiple nutrient components. In best cases these fortified media achieve 5-12

folds of increase over the batch (Table 3). The feeding strategies exploit the combined effects of manipulating both nutrient feed composition and environmental conditions to increase culture longevity and specific secretion rates. The specific rate of the antibody production of cell line selected will also have a profound effect on the final titer. The specific rates for mammalian cells range from 2-80 pg/cell/day (Savinell et al 1989). The reasons for our fed-batch process to have lesser yield were, the cell line used for the study has specific production rate of 3 pg/cell day which is at the lower end of the general mammalian production cell lines. The medium developed in the batch conditions was not completely optimal and from Table 3, it is shown that minimum titer in the batch conditions in literature was around 100 mg/L whereas the titer in our batch medium was just 22 mg/L. The same cell line in commercially ava lable media produced 35 mg/L (Mark et al., 2004) in batch conditions, which indicates that along with the cell line, media is also having an effect on antibody production And in the optimal fed-batch process the total time of culture span was just 254 hr, and the viability started dropping around 210 hr. But to get higher yields culture should span for longer time, where cells would be in the stationary phase and all cell reserves would be directed towards antibody production rather than for increasing the cell density.

Conclusions

The dual-substrate controlled fed-batch culture reduces the formation of the inhibitory products, and increases the yields of cell biomass and antibody concentrations. The offline measurements of the nutrient concentrations in the cultures were relatively close to expected values. The optimal fed-batch process yielded a 44.6% and 31% improvement in cell density and antibody production, respectively, over the batch process. The fed-batch technique offers an effective method of reducing ammonium formation, a necessary prerequisite for obtaining higher cell densities and higher productions during growth Satisfactory results can be achieved by developing a simple and novel feeding protocol and by use of a feed medium (without glucose, added as a different feed) concentrated in glutamine and essential amino acids. This study shows that there is potential for further growth and antibody production in a combined fed-batch culture Additional research should be conducted to identify the growth rate limiting factors through amino acid analysis.

List of Tables: Table 1:

Components	Composition (mg/L)	Components	Composition (mg/L)
CaCl2 (anhyd)	99.1	Biotin	0.02
CuSO4*5H2O	0 00125	D-Ca Pantothenate	4
FeSO4*7H2O	0 415	Choline Chloride	18
KCl	276 8	Folic Acid	4
MgCl2 (anhyd.)	28 61	i-Inositol	25 2
NaCl	5500	Niacinamide	4
NaHCO3	2100	Pyridoxine HCl	4
Na2HPO4 (anhyd)	71	Riboflavin	0 4
Na2HPO4*H2O	62 5	Thiamine HCl	4
ZnSO4*7H2O	0.43	Vitamin B12	1 413
KNO3	0 038	Insulin	10
MgSO4 (anhyd)	50 8	Transferrin	5 5

Na2SeO3	0 0085	Sodium selenite	0.0134
D-glucose	**4000**	Arachidonic acid	0.014
Linoleic Acid	0 04	cholestrol	1 54
Lipoic Acid	0 105	DL-alpha-tocopherol-acetate	0 49
Phenol Red	8 1	Linoleic acid	0.07
Putrescine 2HCl	0 0805	linolenic acid	0 07
Sodium Pyruvate	110	myristic acid	0 07
HEPES	2979	oleic acid	0 07
L-Alanine	4 45	palitoelic acid	0 07
L- Arginine*HCl	410 8	palmitic acid	0.07
L-Asparagine*H2O	6 6	stearic acid	0.07
L-Aspartic Acid	6 65	Tween 80	15.4
L-Cystine*2HCl	78	pluronic	0.10%
L-Glutamic Acid	7 35		
L-Glutamine	**365**		
Glycine	3.75		
L-Histidine*HCl*H2O	136 5		
L-Isoleucine	170.3		
L-Leucine	170 3		
L-Lysine*HCl	235 6		
L-Methionine	49 07		
L-Phenylalanine	107 25		
L-Proline	5 75		
L-Serine	5 25		
L-Threonine	154 7		
L-Tryptophan	33.15		
L-Tyrosine*2Na*2H2O	117		
L-Valine	152.1		

Table 1: Basal media used in fed batch studies (optimized media from batch culture)

Table 2:

Essential amino acids(10X)	Concentration (mg/L)
L-arginine	1264
L-Cystine	240
L-Histidine*HCl*H2O	420
L-Isoleucine	524
L-Leucine	524
L-Lysine HCl	725
L-Methionine	151
L-Phenyl alanine	330
L-Threonine	476
Ltryptophan	102
L-Tyrosine	360
L-Valine	468
L-Glutamine	25 mM

Table 2: FM1, same as the basal medium without glucose and sodium chloride (component concentrations other than EAA and glutamine are given in Table 1), concentrated in L-glutamine, and essential amino acids. FM2 and FM3 are the same as FM1, except that glutamine is concentrated to 7.5 mM and 15 mM, respectively

Table 3:

Feed Composition	Cell line	Batch	Fedbatch	Name	Increase (folds)
Complete Basal Medium	GS-transfected NSO myeloma (unamplified)	100	365	Bibila, 1994a	3 65
Complete Basal Medium	GS-transfected NSO myeloma (amplified)	142	1000	Bibila, 1994a	7
Glucose+Glutamine	Mouse hybridoma (2c3 1)	56 25	450	Jo, 1992	
Glutamine	Mouse hybridoma (TSH-5.07)	25 17	70 5	Franek, 1991	2 8
Glucose+Glutamine	GS-transfected NSO myeloma (amplified)	53 8	350	Brown, 1992	6 5
Glucose+Glutamine	Mouse Hybridoma	152 6	290	Reuveny, 1986	1.9
Glutamine	Mouse Hybridoma 9.2 27	50	250	Flickinger, 1990	5
Amino acids	GS-transfected NSO myeloma (amplified)	77 7	140	Robinson, 1994a	1 8
Amino acids	GS-transfected NSO myeloma (unamplified)	225	900	Robinson, 1994a	4
amino acids+vitamins+serum	hybridoma ATCC HB32	35	140	Luan, 1987b	4
Serum free multi nutrient feeds	mouse hybridoma CRL 1606	50	550	Xie 1994 a,b	11
Serum free multi nutrient feeds	mouse hybridoma	50	200	Noe, 1993	4
Serum free multi nutrient feeds	heterotnoma	100	750	Maiorella 1992a	7 5
Serum free multi nutrient feeds	GS-transfected NSO myeloma (amplified)	344	895	Hassel, 1992	2.6
Serum free multi nutrient feeds	GS-transfected NSO myeloma (amplified)	350	560	Hassel, 1992	1 6
Serum free multi nutrient feeds	GS-transfected NSO myeloma (unamplified)	101 7	865	Robinson, 1994a	8 5
Serum free multi nutrient feeds	GS-transfected NSO myeloma (amplified)	144	1800	Robinson, 1994a	12 5
Serum free multi nutrient feeds	GS-transfected CHO (unamplified)	110		Hassel, 1992	
Serum free multi nutrient feeds	GS-transfected CHO (amplified)	250		Hassel, 1992	
Serum free multi nutrient feeds	SP2/0	98 6	750	Paul W. Sauer, 2000	7.6

Table 3: Monoclonal antibody production (mg/L) in fed batch culture from literature

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Figure 1: Plasmid pS25 containing Light Chain (LC) and Heavy Chain (HC) of s25 antibody against Botulinum Neurotoxin-A, with resistance markers Neomycin, and Amplicillin, and DHFR as a selectable marker.

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Figure 3 The concentration profiles of the nutrients glutamine and glucose of the three sets of experiments. Figures (a), (b), and (c) of the first set, (d),(e), and (f) of the second set, and (g) (h), and (i) of the third set represent cultures controlled at 0, 0.5, and 2 mmol/L, respectively.

Figure 4: Results of total viable cell density and antibody production of cultures controlled at zero, 0.5, and 2 mmol/L in the first set of experiments. Fig. (a) represents the viable cell densities and Fig (b) represents antibody production in reactors

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Figure 6: Results of total viable cell density and antibody production of cultures controlled at zero, 0.5, and 2 mmol/L in the third set of experiments. Fig. (a) represents the viable cell densities, and Fig. (b) represents antibody production in reactors.

Figure 7: Major inhibitory metabolite and total ammonia production rate (mmol/cell/hr) over time in cultures controlled at zero, 0.5, and 2 mmol/L. Figs. (a), (b), and (c) represent the first, second, and third set of experiments, respectively.

Figure: 1

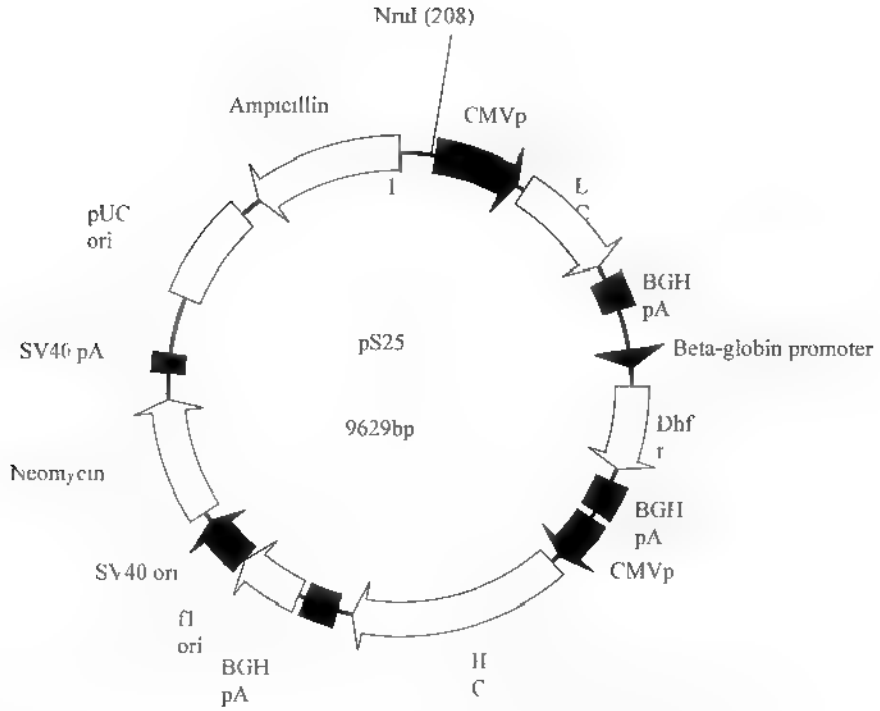
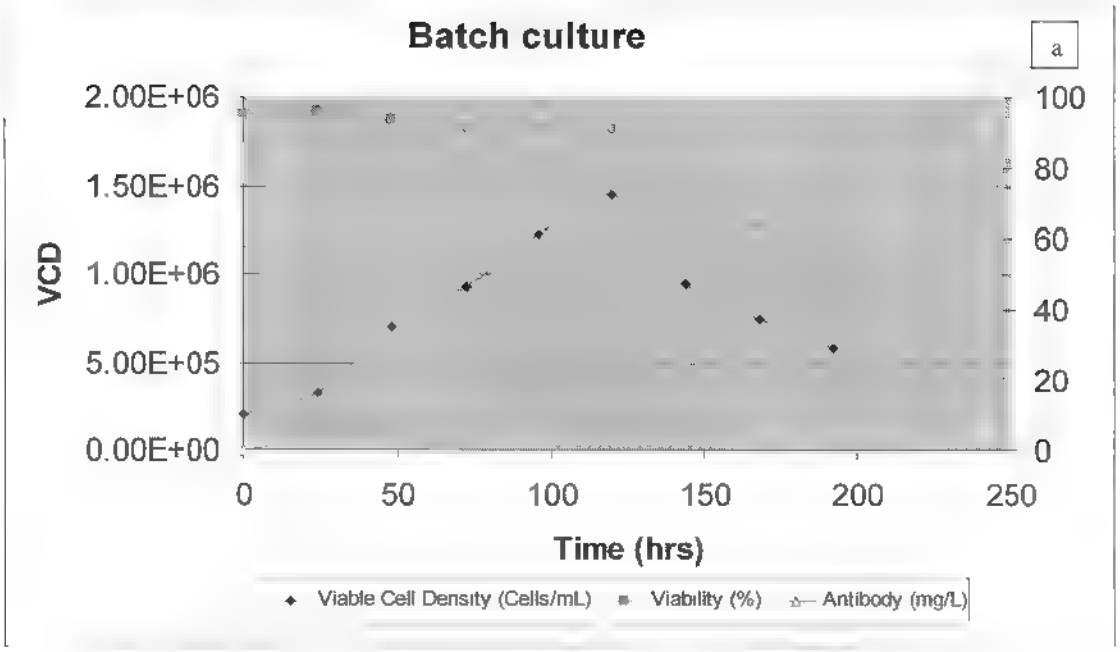


Figure 1: Plasmid pS25 containing Light Chain (LC) and Heavy Chain (HC) of s25 antibody against Botulinum Neurotoxin-A, with resistance markers Neomycin, Ampicillin, and DHFR as a selectable marker

Figure 2:



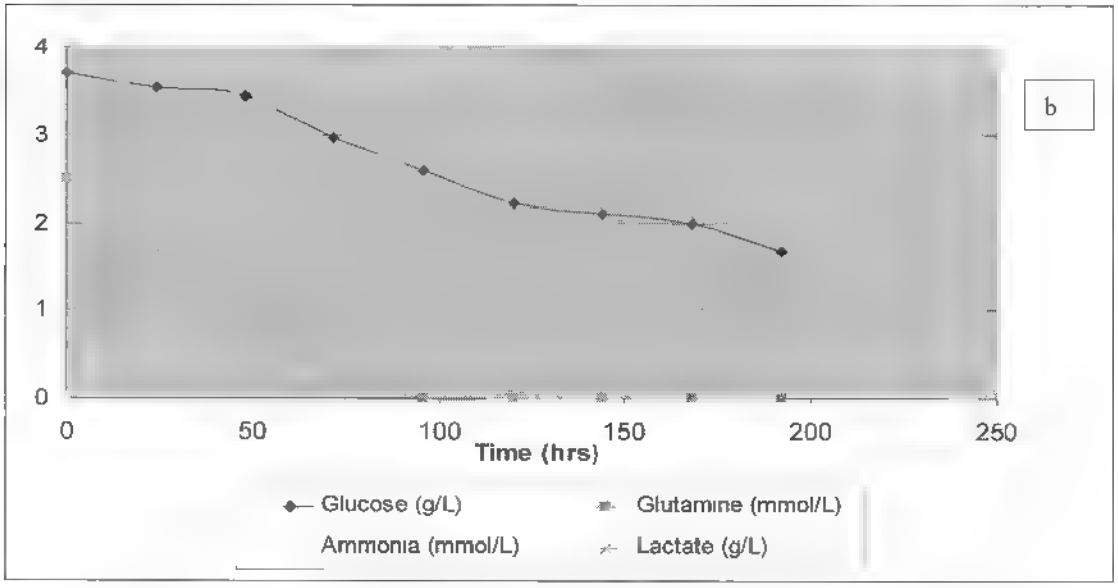
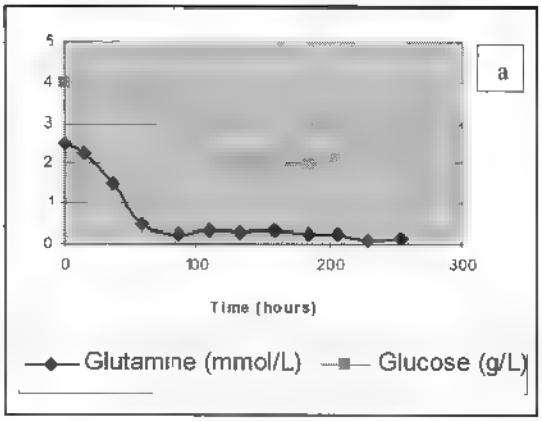


Figure 2: Results of batch culture (CHO-optimal) medium Fig. (a) represents the viable cell Density (Cells/mL), viability (%), and antibody concentration (mg/L). Fig (b) represents the nutrients glucose, glutamine consumed and ammonia, lactate by-product concentrations produced, respectively, over time.

Figure 3: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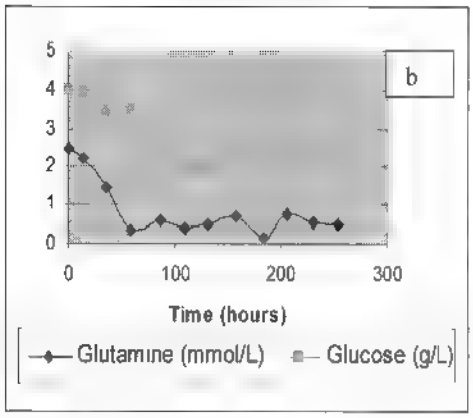
Time (hours)
Glutamine (mmol/L)
Glucose (g/L)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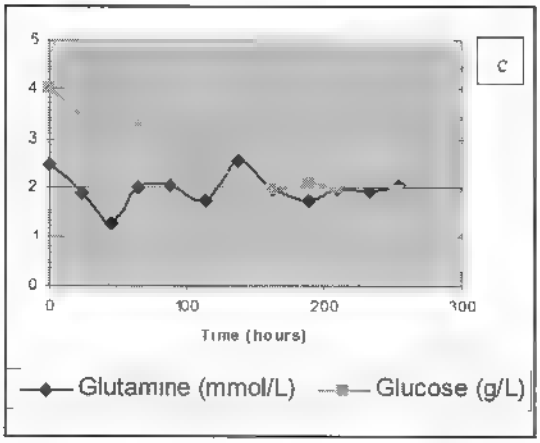
Glutamine (mmol/L)
Glucose (g/L)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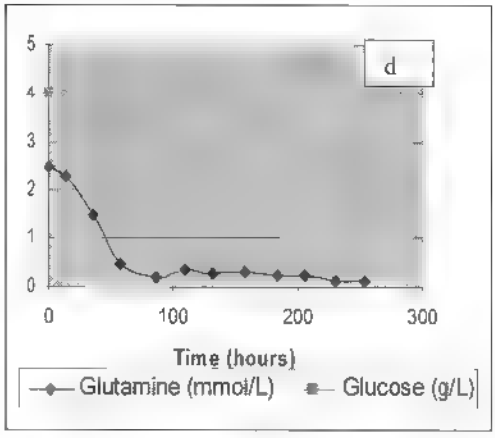
Time (hours)
Glutamine (mmol/L)
Glucose (g/L)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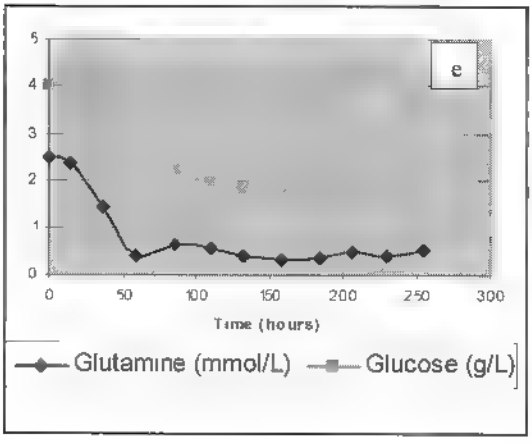
Time (hours)
Glutamine (mmol/L)
Glucose (g/L)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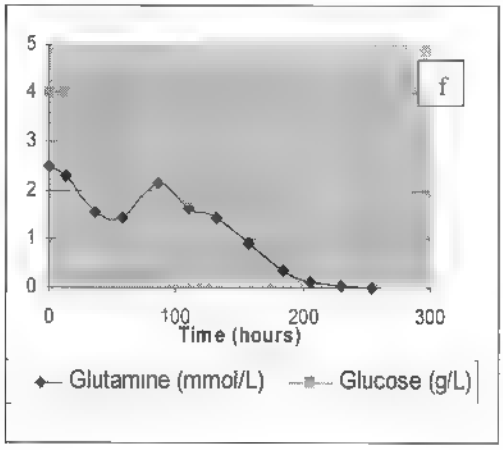
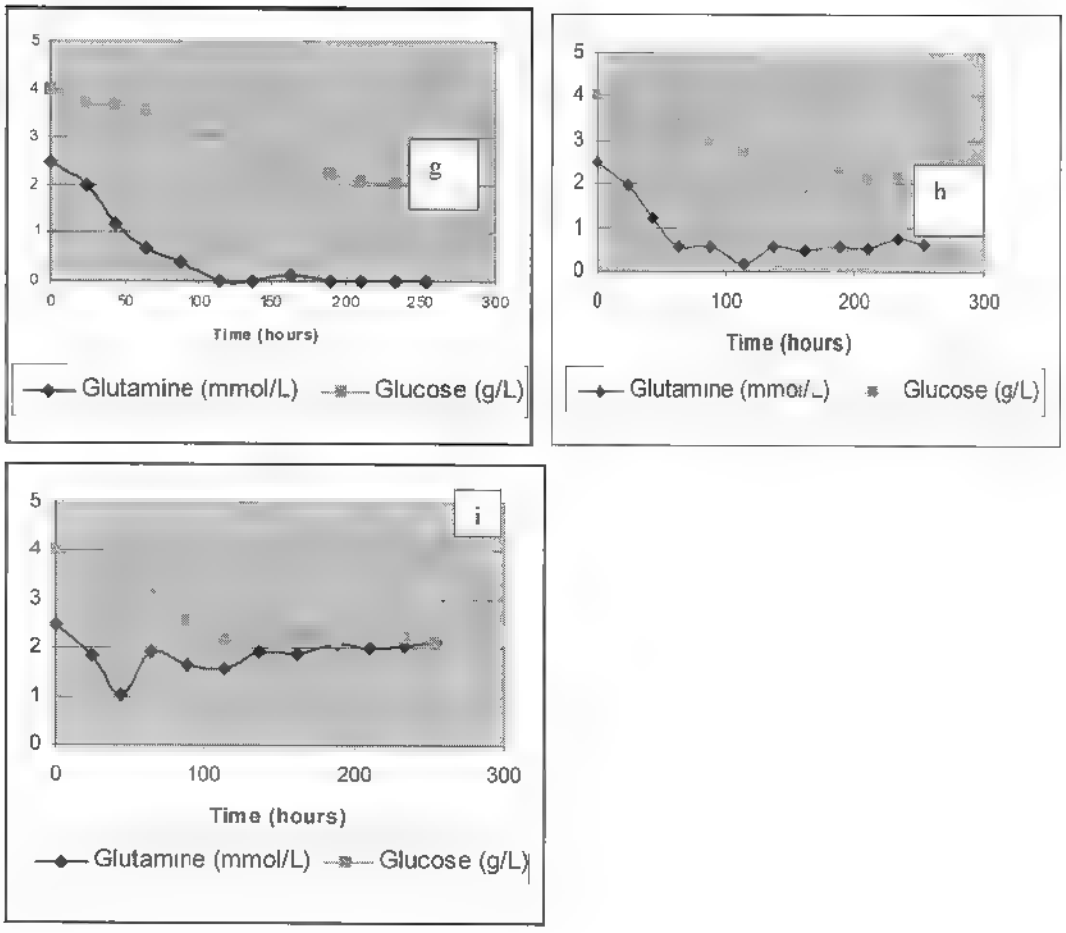
Time (hours)
Glutamine (mmol/L)
Glucose (g/L)


f
Time (hours)
Glutamine (mmol/L)
Glucose (g/L)



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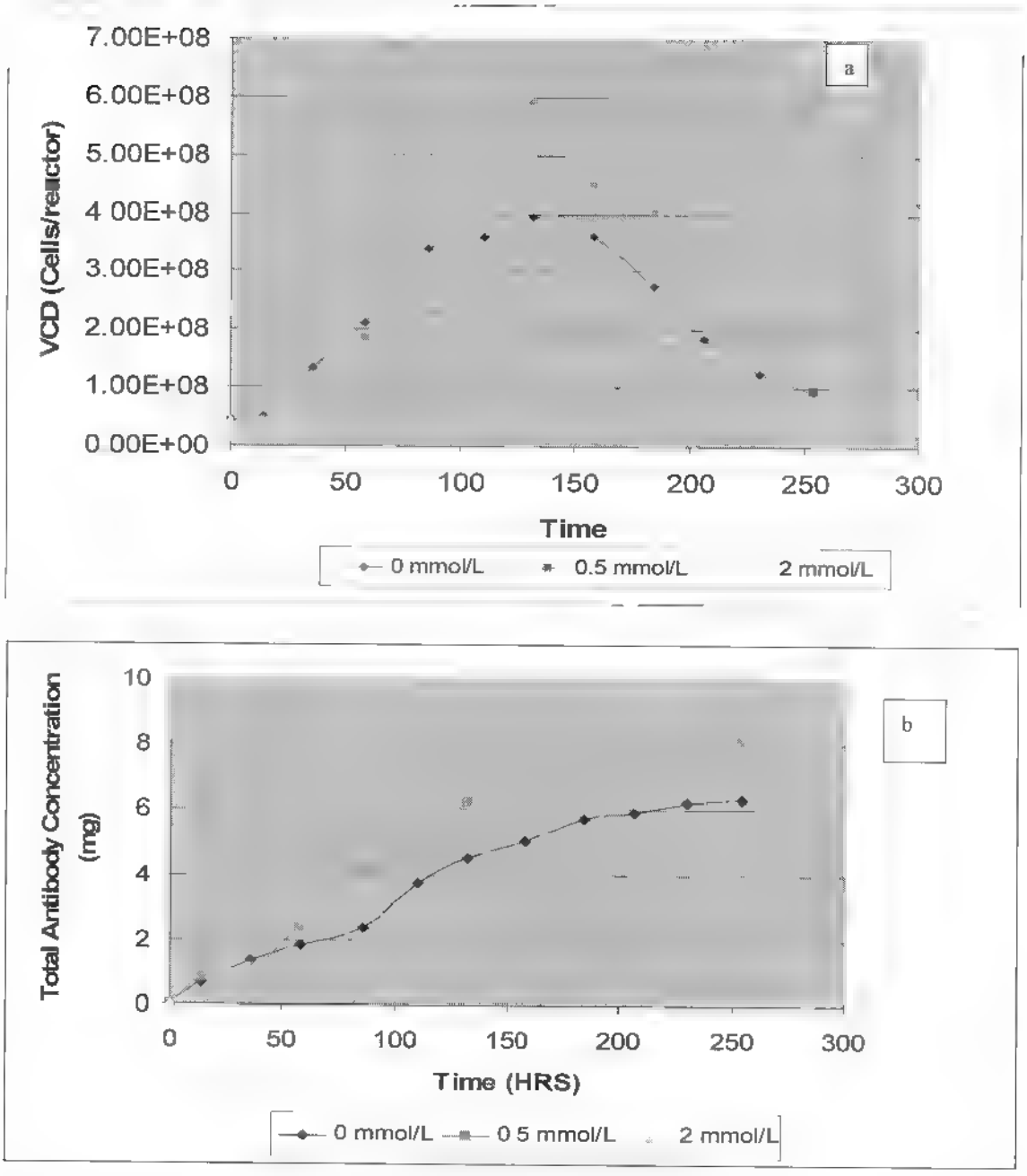

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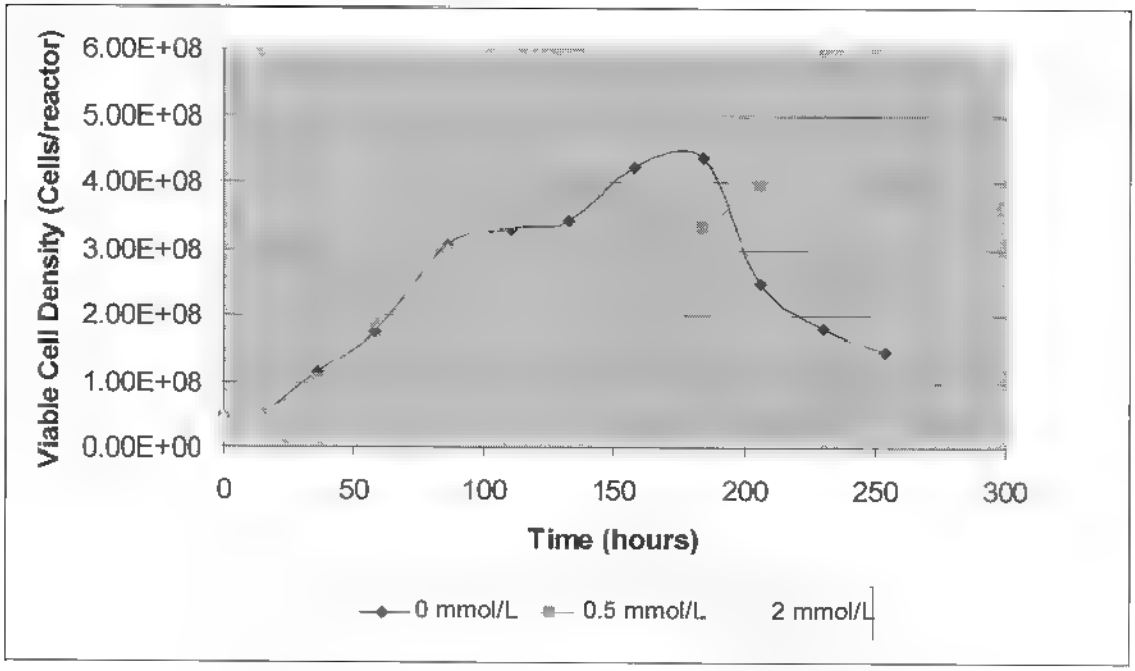
Figure 5:



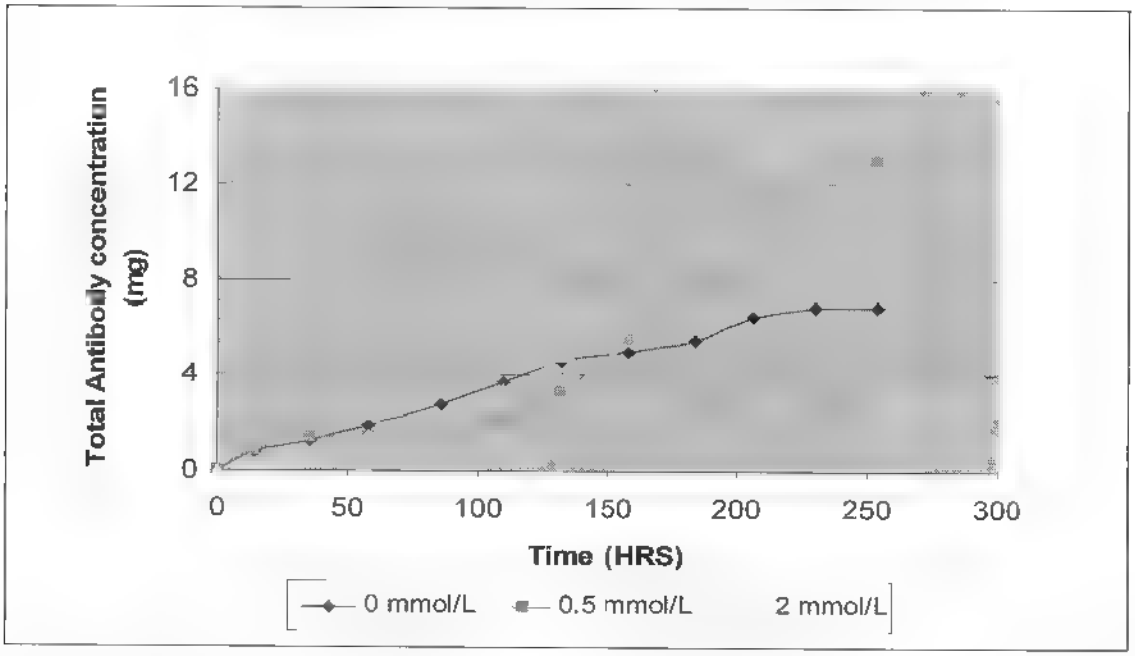
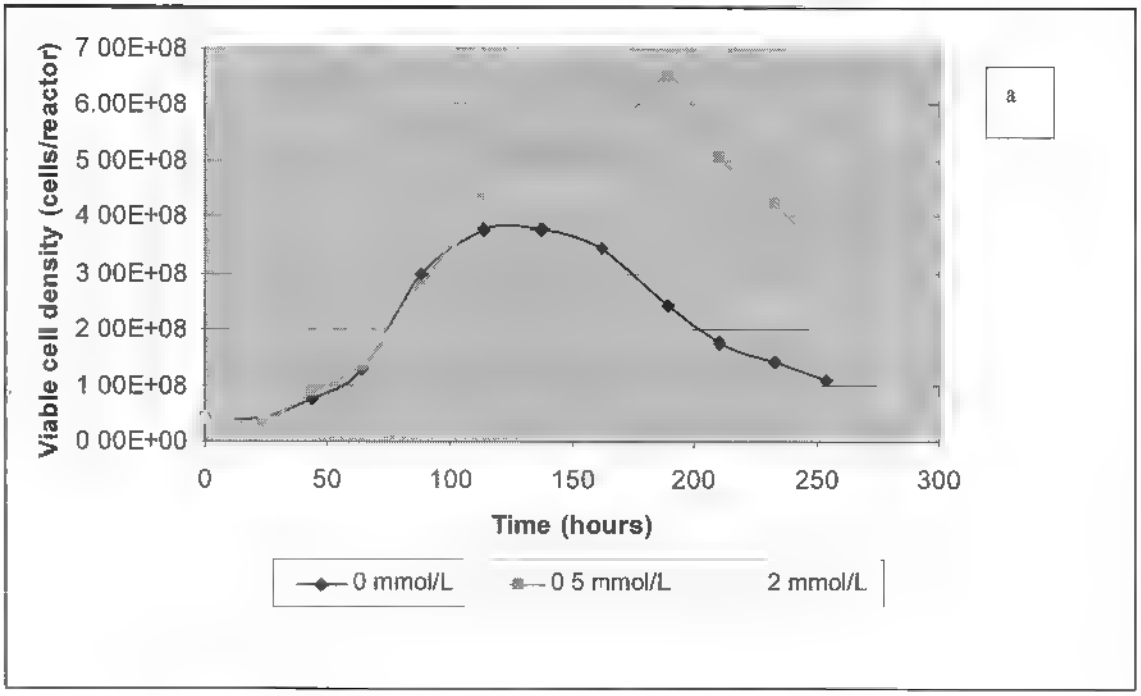


Figure 5 Results of total viable cell density and antibody production of cultures controlled at zero, 0.5, and 2 mmol/L in the second set of experiments Fig (a) represents the viable cell densities, and Fig. (b) represents antibody production in reactors.

Figure 6:



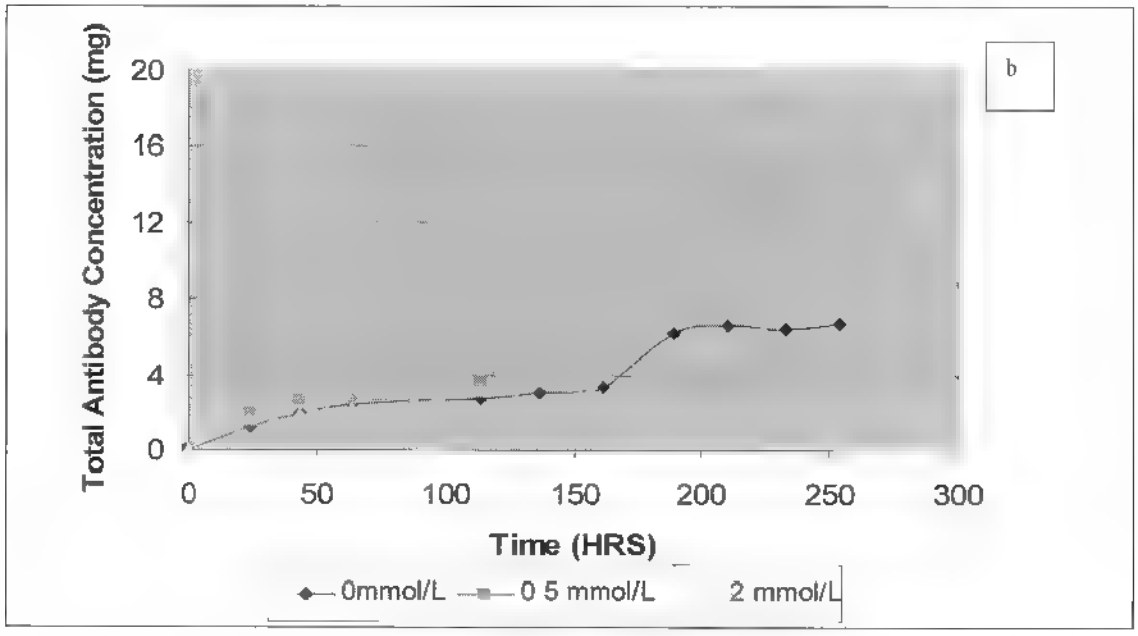
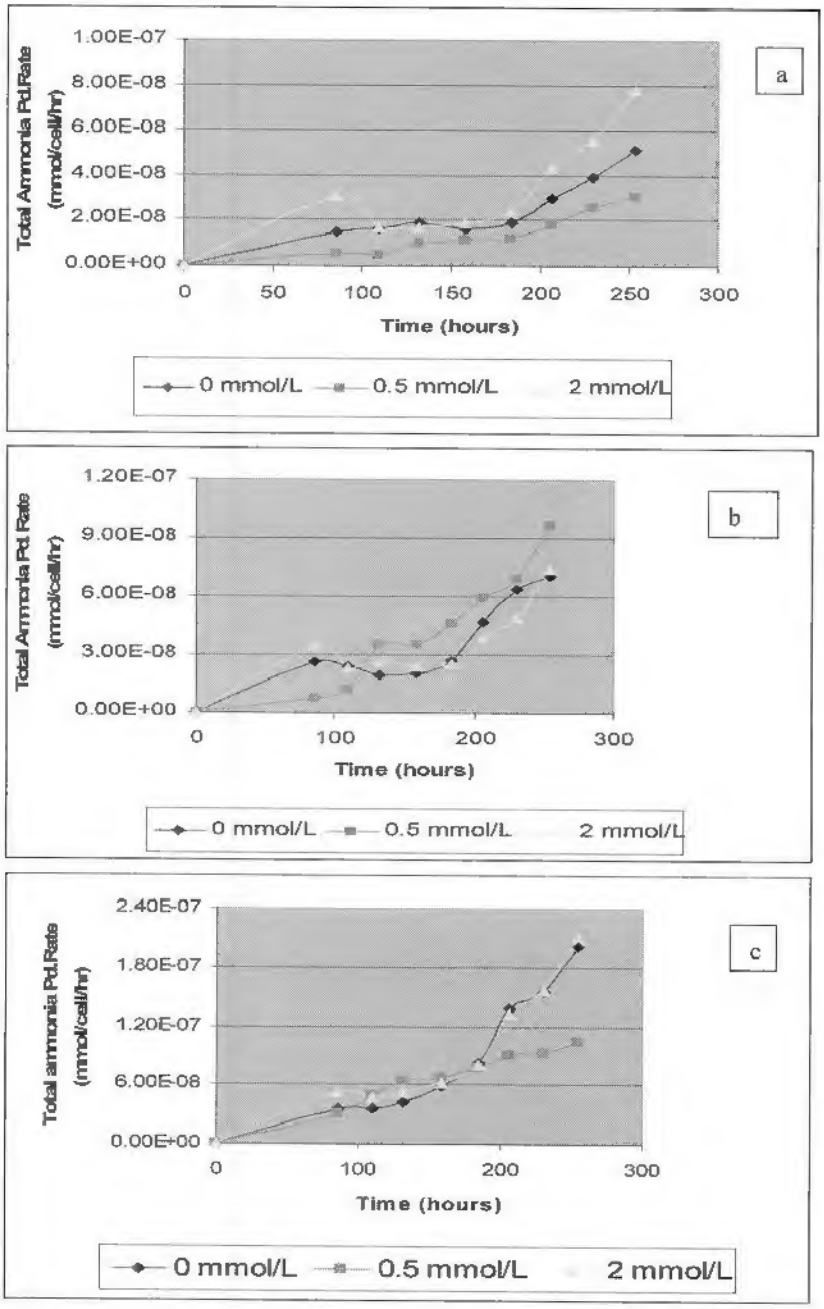


Figure 6 Results of total viable cell density and antibody production of cultures controlled at zero, 0.5, and 2 mmol/L in the third set of experiments Fig (a) represents the viable cell densities, and Fig (b) represents antibody production in reactors.

Figure 7:



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Key Research Accomplishments

- Construction of the shell space on third floor is completed, expect for QC Microbiology and Master Cell Banking Suite, which should be completed by Fall 2005.
- Construction of new and improved plasmids for expression of antibodies in CHO cells
- A preliminary optimized media has been identified and a manuscript submitted.
- Preliminary studies completed on optimization of a fed-batch process for optimization of CHO expression.